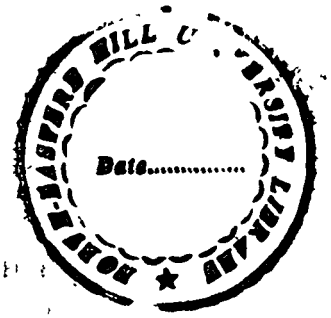
Extent of DNA damage induction by raw betel-nut extract in mammalian cells and loss of heterozygosity at chromosome 9p in oral and esophageal carcinoma in raw betel-nut chewers.



By

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DECLARATION

I, Allen J. Freddy, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North Eastern Hill University for the degree of Doctor of Philosophy in Zoology.

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Dedicated to
Dad, Mom and Haans

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Allen

CONTENTS

ABBREVIATIONS

AAS	Atomic absorption spectrometer
AEBN	Aqueous extract of betel nut
AGT	Average generation time
APS	Ammonium persulphate
ARC	Arecoline
ARF	Alternate reading frame
ASNAs	Areca nut specific nitrosamines
BMC	Bone marrow cells
BN	Betel nut
BNE	Betel nut extract
BQ	Betel quid
BSO	L-Buthionine-S-R-Sulphoximine
BUdR	5-Bromodeoxyuridine
CAs	Chromosomal aberrations
CDKN2A	Cyclin dependant kinase 2A
CDK's	Cyclin-dependent kinases
CIN	Chromosomal instability
CKI	Cyclin dependant kinase inhibitor
DNA	Deoxyribonucleic acid
dNTP	di-nucleotide triphosphate
DTT	1, 4- Ditheithretol
EC	Esophageal carcinoma
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
FPG	Fluoresce plus Giemsa staining
GSH	Glutathione
HNSCC	Head and neck squamous cell carcinoma
HPBLs	Human peripheral blood lymphocytes
i.p	Intra peritoneal
INK4	Inhibitor of kinase 4
KCl	Potassium chloride
LOH	Loss of heterozygosity
MI	Mitotic index
MNPN	3-(methyl-N-nitrosamino) propionitrile
MSI	Microsatellite instability
MTS1	Multiple tumor suppressor-1
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NET	Sodium chloride, ethylene diamine tetra acetic acid, tris

NG	N-nitrosoguvacoline
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNK	Polynucleotide kinase
Rb	Retinoblastoma
ROS	Reactive oxygen species
RPMI	Rosewell park memorial institute
SCC	Squamous cell carcinoma
SCE	Sister chromatid exchange
SOD	Super oxide dismutase
STRs	Short tandem repeats
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris, ethylene diamine tetra acetic acid
TSG	Tumor suppressor gene

GENERAL INTRODUCTION

Environment encompasses all non-genetic factors such as diet, lifestyle and infectious agents. In this broad sense, the environment is implicated in the causation of the majority of human cancers, as has been demonstrated since the 1960s (Doll, 1969). Such a broad meaning of the word environment is assumed when referring to gene environment interactions. On the other hand, environmental factors can include only the (natural or man-made) agents and circumstances encountered by humans in their daily life, upon which they have no or limited personal control. In this context, environmental factors are restricted to air, water, soil and food pollutants, including physical pollutants such as sources of ionizing radiation. These ambiguities in the terminology and the inconsistencies in the use of the vocabulary by cancer researchers contribute to public confusion regarding the role of environment in cancer. A distinction relevant to cancer prevention may be made between factors related to personal behaviors, lifestyle (e.g. tobacco smoking, chewing of betel quid and alcohol drinking), involuntary exposures, such as those linked to air, water, soil or food pollutants, and occupation. It would be preferable to abandon the term environment and to use terms such as non-genetic or modifiable determinants of disease (broad sense of environment) and pollutants (narrow sense). The oral cavity and esophagus combined is the sixth commonest site of cancer in both sexes. In many countries the mortality rate is increasing among younger men. A causal role in the etiology of both oral and esophageal cancer has been established for tobacco, both smoking and chewing, separately and in conjunction with betel quid chewing; with alcohol consumption and, less certainly, with other factors such as poor oral hygiene, nutritional factors and certain occupational exposures. In Western countries, there is convincing evidence that a large attributable risk can be ascribed to the joint habits of cigarette smoking and alcohol consumption. In Asian countries, a high attributable risk can be ascribed to cigarette smoking and betel-nut chewing. Cancer of the oral cavity is an important form of cancer and one for which practical prospects for prevention already exists. However the prognosis of esophageal cancer is very poor, its 5 years survival rate is only about 10% for the patients at late or advanced stage. The early characteristic of the

subjects predisposed to esophageal cancer is the abnormal proliferation of epithelial cells, morphologically manifested as basal cell hyperplasia, dysplasia and carcinoma in situ, which could be considered as precancerous lesions of esophageal cancer.

Related to its many functions, the oral cavity contains several different types of stratified squamous epithelia, including those classified as nonkeratinized, parakeratinized, and orthokeratinized (Burkhart and Maerker 1981). Regional variation and heterogeneity within each type of epithelium also include glandular epithelium (salivary glands) and taste buds, the latter on the dorsal and lateral tongue. Primarily nonkeratinized epithelium provides a lining in the cheeks, lips, floor of mouth, ventral aspect of the tongue, soft palate, and upper and lower vestibular sulci. Parakeratinized and orthokeratinized epithelium lines the hard palate and the mucosa that surrounds the teeth (attached gingiva). The dorsal tongue and gingival margin are such zones. The basement membrane zone, the papilla and reticular zones of the lamina propria, and beneath these, the submucosa, typically support the various oral epithelia. The very similar structure of the oral epithelium and the epidermis, including the squamous nature of both and the generation of a surface barrier, naturally implies that many of the research results with epidermal keratinocytes are also applicable to the oral epithelium.

Damage to genetic material could result in a number of immediate and long-term effects, finally leading to cancer. A search for possible voluntary exposure that could be used as a model for investigating human carcinogenesis led to consideration of betel nut chewing. Investigations of this possibility offer an opportunity both to study this specific hypothesis, investigate and to develop approaches for similar studies of other exposures along with betel nut. This approach could be further described as the use of laboratory tests to define exposures, susceptibility factors, and the pathophysiological sequence of events linking exposure and disease in epidemiologic studies of chronic diseases. Carcinogenesis studies in experimental animal models indicate that an early event for many of the chemical carcinogens is to cause damage to the genetic material. Oral cancer is one of the most prevalent cancers in South and Southeast Asian

countries. There are strong indications for an association of the habit of betel quid chewing with cancers of the mouth, oropharyngeal cavity, and upper parts of the digestive tract (Dave et al., 1992; IARC Monograph 1985). Moreover, chewing and smoking habits interact synergistically for these cancers (Jayant et al., 1997). Although some pathological (Pindborg, 1980), epidemiological (Hirayama 1966) and genetic studies on oral cancer and precancer have been previously reported, the incidence of oral cancer is still very high in these countries and only a few efforts have been carried out for its prevention.

Oral cancer was described in the Sushruta Samhita, a treatise on Indian surgery written in Sanskrit around 600 B.C. Literary references to the habit of chewing betel quid (betel leaf, areca nut and lime) in India are at least 2,000 years old. Tobacco was introduced around the sixteenth century. It is estimated that at least 600 million individuals consume areca nuts in one form or another worldwide. The habit is now widespread in Southeast Asia and the South Pacific islands and in people of Indian origin elsewhere in the world. The betel quid chewing habit is found all over the world wherever Indians have settled. The major areca nut alkaloids are arecoline, arecaidine, arecolidine, guvacoline and guvacine (IARC Monograph, 1985). Arecoline (1,2,4,5,-tetrahydro-1-methyl-pyridine- carboxylic acid; molecular weight 155.19) is the most abundant alkaloid of areca. These alkaloids undergo nitrosation and give rise to N-nitrosamines (Hoffmann et al., 1994). It has been suggested that metabolic activation may involve the cytochrome p450 system (Sundqvist et al., 1991; Wary and Sharan, 1991). The nitrosation of arecoline may produce a variety of betel quid-specific nitrosamines which may interact with DNA, proteins or other targets forming adduct to exert its carcinogenic activity. The introduction of tobacco from European countries reinforced this practice, and now almost all habitually chewed betel quid include tobacco. A comparison of the carcinogenicity of the habit of chewing betel quid with and without lime has been attempted through a reassessment of the available epidemiological evidence on the etiology of oral cancer and pre-cancer. Slaked lime included in betel quid causes inflammation in the sub mucosal area. Calcium hydroxide content of lime in the presence of the areca nut is primarily responsible

for the formation of reactive oxygen species that might cause oxidative damage in the DNA of buccal mucosa cells in betel quid chewers (Nair et al., 1990).

Ever since the discovery of glutathione (GSH) in the early thirties, this tripeptide has been a subject of continual interest. This versatile antioxidant is found to be involved in various biological functions (Meister, 1983). Although this tripeptide can exist in both reduced (sulphydryl) and oxidized (disulphide) form, it is maintained *in vivo* predominantly in the former state, however the, balance is maintained through the action of equally ubiquitous enzyme Glutathione Reductase (Knox, 1960). GSH is found throughout the cell, with the bulk in cytoplasm, subcellular particles, such as nucleus, mitochondria having smaller amounts (Bigalow and Tutle 1993). It plays an important role in regulation of cellular proliferation and cellular defense against radiation (Chatterjee and Jacob-Raman 1986) and various xenobiotics (Syng-ai et al., 2002). Most workers have used BSO for studies involving the modulation of GSH levels in cells. BSO inhibits GSH synthesis and very low levels of GSH can be obtained over a relatively short period without appreciable toxicity. The most useful approach to deplete the level of endogenous GSH is the treatment with BSO, a specific inhibitor of γ-glutamylcysteine syhthetase, the enzyme that catalyzes the step of GSH synthesis (Griffith and Meister 1978). BSO is a potent selective inhibitor of GSH synthesis that is highly effective both *in vivo* and *in vitro* without showing any side effect (Griffith and Meister 1978). Depletion of GSH by treatment with BSO sensitizes the cells to toxic effects of heavy metals (Singhal et al., 1987, Naganuma et al., 1990), nitrogen mustard (Suzakake et al., 1982, Suzakake et al., 1983), radiation and cisplatin (Edgren and Revez 1987) but not radiomimetic drugs like bleomycin (Chatterjee et al., 1989).

The precancerous lesions are instable, they can develop to cancer, or stay for a couple of years without any changes, or even return to normal. What is the most important factor to decide the precancerous lesions to developing different directions, especially in those with a similar morphology? What is the key point to induce mild precancerous lesions to develop cancer? Our assumption is that there

exist different molecular changes in precancerous lesions with a similar morphology. To characterize the molecular changes in carcinogenesis of both oral and esophageal cancers, the mechanism of these cancers could be elucidated and the biomarkers for early diagnosis and mass survey of high-risk populations could be established. It has been shown that heavy chewers of betel-nut (BN) in the Meghalaya state show higher DNA damage, delay in cell kinetics, p53 expression and lower GSH-level than non-chewers (Kumpawat and Chatterjee 2003).

The north-east Indian variety of BN is raw, wet and consumed unprocessed and chewed along with betel-leaf and slaked lime. The constituents of this nut show higher alkaloids, polyphenol and tannins compared to the dried one (Sharan 1996) and is locally known as 'Kwai'. (Stich et al. 1983) demonstrated the genotoxic potentiality of saliva of kwai chewers of the tribal population of Meghalaya state of the north-eastern region of India in Chinese hamster ovary cells. The average age of onset of chewing among tribes was about 12 years, and at 35 years of age and older, the frequency of oral carcinoma rose significantly.

Microsatellites are repeated DNA sequences scattered widely within the genomes and closely linked with many important genes (Shamoo, 2003). In recent years many researchers have indicated that the alteration of microsatellite DNA is one of the important markers, which could induce normal cells to undergo immortal and neoplastic transformations. It was reported that an extensive loss of microsatellite DNA was discovered in many tumors such as colon cancer. Some microsatellite loci often exist in the hot spots of loss of heterozygosity at a high frequency in some specific malignancies. Tumor suppressor genes, which are associated with the development and progression of tumors, may harbor in the vicinity of these hot spots.

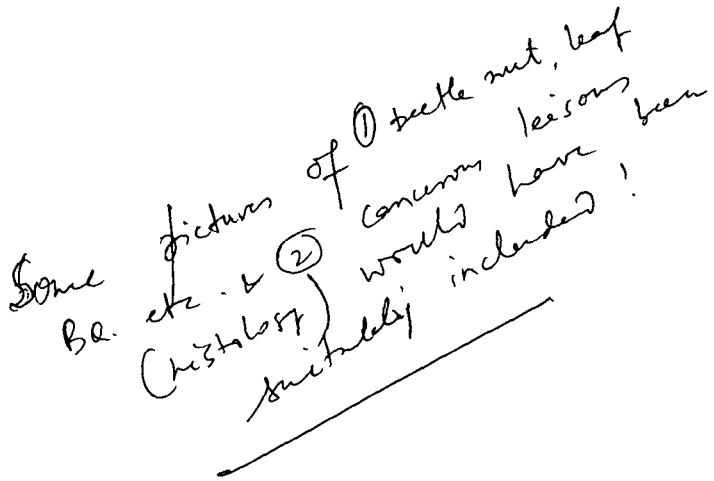
Histology of the tumor samples, the current gold standard for assessing the risk, is reasonably effective in judging the malignant risk of high-grade pre-invasive lesions. It is, however, a poor predictor for lesions without dysplasia, or with minimal dysplasia, as only a few of these lesions will progress to cancer. This poses an enormous dilemma for clinicians as to whether these lesions should be aggressively treated or not. Recent studies show that loss of specific chromosomal

regions that contain known or presumptive tumor suppressor genes is an early predictor of subsequent progression of oral premalignant lesions. Incorporation of loss of heterozygosity (LOH) findings into staging of oral premalignancy could improve our ability to identify and manage high-risk premalignant lesions, particularly those with relatively benign histology but high-risk genetic changes (high-risk LOH pattern).

It is true that from the background we have some idea about the mechanism of action of the betel-nut extract; however, we will have to know some more things which will be helpful to understand about its action. Therefore, we have primarily two objectives to investigate and they are:

- Evaluation of the extent of initial DNA damage / lesions induced by raw betel nut in mammalian cells.

- Identification of critical regions of Loss of Heterozygosity (LOH) by deletion mapping by PCR based techniques using appropriate microsatellite markers in 9p chromosome arm in order to know the status of p16 gene in tumor samples collected from raw-BNE chewers.



Chapter I

Extent of DNA damage caused by Betel nut with or without lime

Introduction.

Like various psychoactive plants of antiquity betel nut has been used by mankind since the pre Christian era. The earliest document is Maharamsa, a Ceylonese document from 540BC where there is a mention of the use of areca nut. In India in the early Christian era, betel nut use was frequently referred to in Sanskrit medical literature as well as in Hindu and Buddhist writings. BNE in those early days was considered to have both medical and psychosomatic effects, for example as a breath freshener, digestive agent, and also anti helminthic proprieties. Areca nut is the seed of the tall, slender areca palm (areca catechu), native to the fringes of the Indian and west Pacific Oceans. It is thought to have originated on the Malay Peninsula. It is one of the oldest known masticatory used by Asians (Raghavan and Baruah, 1958). Areca nut is one of the most widely used psychoactive substances with several hundred million users worldwide, predominantly in southern Asia. Around 600 million people in South-east Asian countries are addicted to areca (Boucher et al., 2002). Chewing of betel quid and areca nut is an ancient custom in several parts of South East Asia, the south Pacific Islands and Taiwan, and this practice is deeply entrenched in the culture of the population. It arrived in India in the 16th century. The habit of chewing betel quid, containing fresh, dried or cured areca nut, catechu, slaked lime and flavoring ingredients wrapped in betel leaf is widespread in India, Pakistan, Bangladesh and Sri Lanka and in migrant populations coming from these regions. Potent carcinogenic agents have been derived from areca nut in particular betel nut specific nitrosamines. Betel users discovered that the addition of lime helps to extract the vital essence of the nut, the catalytic lime is either powder (calcium oxide) or paste (calcium hydroxide). The evidence for the carcinogenicity of humans to betel quid with tobacco was evaluated as sufficient; the evidence for betel quid without tobacco was evaluated as inadequate. The chewing of betel nut has been established as the principal etiological factor for the high incidence of oral cancer in India and some other Asian countries. Epidemiological studies have elucidated that the habit of chewing betel quid (BQ) is associated with an increased risk of oral submucous fibrosis (OSF) and oral cancer (IARC 1985, Kwan 1976). Many more studies now provide evidence for the carcinogenicity of

betel quid without tobacco for oral cancer and for betel quid with tobacco for cancers of the oral cavity, pharynx and esophagus. The Khasis in Meghalaya chew one quarter of the betel nut along with a portion of the betel leaf on which lime has been smeared. As a rule the inclusion of tobacco and spices or other performs are avoided. This unique situation favors an investigation into the length and exposure of the oral mucosa to the chemicals released from the BNE and leaf during the course of the day (Stich et al., 1983). East Khasi Hills a district in Meghalaya of the north-eastern region of India has one of the highest incidence rates of oral and esophageal cancer which is of particular concern. Studies reveal that Head and neck cancers still have a high mortality rate.

Arecoline (ARC), the major alkaloid of the betel nut, is mutagenic in both bacteria and mammalian cells (Shirname et al., 1984). It increases the frequency of micronuclei and chromosomal aberrations in mouse bone marrow cells in vivo (Panigrahi and Rao 1982) and in Chinese hamster ovary cells in vitro. It has also been demonstrated that the frequencies of CAs and sister chromatid exchanges (SCE) in peripheral blood lymphocytes and micronucleate cells in exfoliated cells of buccal mucosa are increased in areca nut chewers (Dave et al., 1992). It has been shown that ARC induced DNA damages is influenced by endogenous GSH level (Deb and Chatterjee 1998) and the aqueous extract of BNE induced DNA damage and reduced the endogenous GSH level (Kumpawat et al., 2003). It was demonstrated that oral administration of ARC in mice induced higher frequency of DNA damages than intraperitoneal injection and the presence of N-acetyl cysteine significantly reduced the effect of ARC (Chatterjee and Deb, 1999). By treating the cells with ARC in anoxic condition and by applying superoxide dismutase it was observed that ARC generates reactive oxygen species that partially contribute to the induction of DNA damages (Kumpawat et al., 2003).

In India, betel nut chewing is an important factor, besides tobacco and alcohol, which is responsible for oral carcinogenesis. Oral Squamous Cell Carcinoma (OSCC) is the 3rd most common malignancy in developing countries and 6th world wide. The average age of onset of chewing among tribes was about 12 years. Stich had measured the length of exposure of the oral and esophageal

mucosa to chemicals, which are released from the raw betel nut, lime and leaf during the course of a day. Assuming an average of 15 min as per chewing period, the oral mucosa would get exposed daily for about 3.5 h to chemicals released from the quid. Among BNE-chewers of 35 yrs of age and older, the frequency of oral carcinoma rises significantly. Thus, in the period between the onset of chewing and the diagnosis of leukoplakia / tumors, the oral mucosa was exposed for about 28,000 h (3.2 yrs) to betel quid extracts.

Composition of Betel-nut

Betel Nut:

Betel nut, the fruit of (*Areca catechu* Linnaeus) is a slender, single-trunked palm that can grow to 30 m (100 ft). Areca palm is native to South Asia and is found throughout South and South East Asia and in several Pacific Islands.

Betel leaf:

A very important associated species is betel pepper vine (*Piper betel*), which is grown for its leaves. The leaves of the betel vine are used as the wrapper when preparing a quid of betel nut with lime, tobacco, or other ingredients.

Lime:

Lime contains 15 major and minor components determined by x-ray fluorescence spectrometry, atomic absorption spectrophotometer (AAS) and combustion methods. Lime is either consumed as paste calcium hydroxide or as powder calcium oxide. The use of lime by betel-quid chewers to achieve an alkaline pH may play a role in the genesis of oral cancer, particularly in areas of the world where no tobacco is used (Atkinson et al., 1964).

Table 1a Constituents of BNE endosperm before curing:

Constituent	Quantity
Tannins	11.4 -26%
Gallotannic acid	18.03%
D-Catechol	3g/800g (0.4%)
Alkaloids	0.15-0.67%
Arecoline	0.7-0.50%
Arecadine	Small quantity
Guvacine	Small quantity
Isoguvacine	Trace quantity
Arecolidine	Minute quantity
Guvacoline	Minute quantity
Fats	1.3-17%
Sitosterol	Trace quantity
Carbohydrates	47.2-84.5%
Non protein nitrogen	0.22-1.6%
Carotene	5 International
Calcium	0.018- 0.05%
Phosphorus	0.13- 2.53%
Iron	1.5-11.6mg/100g(0.002-0.01%)

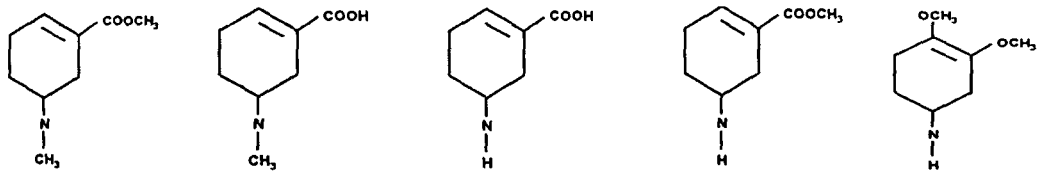
(From Raghavan and Baruah, 1985)

Table 1b Constituent of Lime

Constituent	Quantity
Silicon	<0.01-0.9%
Aluminium	<0.1-0.5%
Iron	0.1-0.21%
Magnesium	0.1-0.59%
Calcium	55-73%
Sodium	0.01-0.99%
Potassium	0.01-0.09%
Titanium	0.01-0.04%
Phosphorus	0.03-0.11%
Manganese	< 0.02-0.32%
Sulfur	0.01-0.69%
Strontium	0.02-1.07%
Carbon	2.4-4.2%

(From H. Bartsch and U.J.Nair)

Alkaloids: Alkaloids are prime suspects for BNE carcinogenesis. Arecoline (1,2,4,5,-tetrahydro-1-methyl-pyridinecaboxylic acid) which is one of the cholinomimetic alkaloid in BNE. Most of the effects of BNE have been attributed to the actions of arecoline. Arecoline a monofunctional alkylating agent loses one of its methyl group during metabolism and binds with the DNA and proteins. The secondary effect of this binding is depurination which may lead to the breakage of the chromosome. The areca alkaloids comprise arecoline, arecadine, guvacoline, and guvacine and betel quid chewers are exposed to these alkaloids, principally arecoline and arecadine. Complex reactions occur during chewing of BNE. In the presence of lime with its high alkalinity, arecoline and guvacoline are hydrolyzed to arecadine and guvacine. The majority of arecoline metabolites are formed after the compound is first hydrolyzed to arecadine. *N*-Oxidation and mercapturic acid formation are the only two pathways of arecoline metabolism not involving prior hydrolysis. Arecadine also undergoes *N*-oxidation and mercapturic acid formation. The principal pathways of arecoline metabolism appear to be hydrolysis to arecadine, *N*-oxidation, together with double-bond reduction of the metabolite arecadine. Both duration and daily frequency of areca use increase the risk of developing cancer, suggesting a dose response relation. The areca-nut-derived nitrosamines, *N*-nitrosoguvacoline and 3-(methylnitroso)propionitrile, have been detected in saliva and are most probably produced *in situ* during betel-quid chewing.



Arecoline **Arecadine** **Guvacine** **Guvacoline** **Arecolidine**

Arjungi (1976) and (Mazumdar 1982) have reviewed the toxic and pharmacological effects of BNE. Aqueous extract of BNE increased GSH content

and decreased protein –SH in liver, kidney and muscle of Swiss albino mice (Shivapurkar and Bhide 1978). Areca-nut chewing has significant effects on the hard and soft tissues of the oral cavity. Areca-nut polyphenols increase cross-linkage of collagen and inhibit the activity of collagenase. Arecoline modulates the activity of matrix metalloproteinases, their tissue inhibitors and lysyl oxidase, which leads to the accumulation of collagen in the fibroblasts of the oral mucosa. This may result in the development of submucous fibrosis. The significant amounts of soluble copper released into the oral cavity by areca-nut chewing may further contribute to the development of this condition. The oral cavity has been described as a mirror that reflects the health of the individual. The mucosa of the mouth and esophagus may appear to differ little from the rest of the moist lining of the gastrointestinal tract, with which it is continuous. The soft tissues of the human oral cavity and esophagus are covered everywhere by a stratifying squamous epithelium (Squiver et al., 1989). The esophagus extends from the upper esophageal sphincter which delineates it from the oropharynx, to the lower esophageal sphincter, representing the junction with the gastric mucosa. Chemical carcinogenesis is now recognized to consist essentially of two steps namely initiation and promotion in most tissues. The first stage initiation results in the binding of the carcinogen to cellular DNA followed by fixation of the alteration during DNA replication. More than 60% of the cancers have environmental factors in their etiology, this generated a lot of interest in chemical carcinogenesis. It is now evident that some chemicals may not be carcinogenic per se but may require metabolic activation. During the last few years metabolic activation of these carcinogens have received attention primarily due to the elucidation of the critical role of biotransformation in chemical carcinogenesis.

Areca nut extract has been found to be both cytotoxic and genotoxic and is widely implicated in the development of oral cancers (Hirayama 1966). The saliva of betel nut chewers has been documented to contain nitrosamines derived from areca nut alkaloids (Dave et al., 1992). Betel-quid or areca-nut chewing results in exposure to areca nut alkaloids, as well as polyphenols, tannins and trace elements. The use of lime by betel quid chewers to achieve an alkaline pH plays a

crucial role in the genesis of oral cancer (Arkinson et al., 1964), particularly in areas where no tobacco is used in the quid. The areca nut specific nitrosamines (ASNAs) N-nitrosoguvacoline (NG) (Wenke et al., 1984; Nair et al., 1987; Stitch et al., 1986) and the carcinogenic 3-(methyl-N-nitrosamino)propionitrile (MNPN) (Prokopezyk et al., 1987) were also detected in the saliva of chewers of BQ without tobacco. ASNAs were not detected in BQ containing areca nut. Nitrosation of BQ with nitrate and thiocynate *in vitro* at neutral pH resulted in the formation of NG (Nair et al., 1985). Nitrosation of arecoline at neutral pH yielded approximately four times more NG than at acidic or alkaline pH (Wang and Peng, 1996). The highest levels of ASNANG were found in the sediment of saliva collected from Taiwanese BQ chewers (Stich et al., 1986).

Biomarkers for monitoring cytogenetic damage

The National Academy of Sciences defines a biomarker or biological marker as a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample (ENTOX/TIWET 1996). The use of biological markers in the evaluation of disease risk has increased markedly in the last decade. Biomarkers are observable endpoints that indicate events in the process leading to disease. Chromosomal alterations have been used as important biological endpoint to study the mutagenic effects of carcinogenic chemicals as well as ionizing radiation. Structural and numerical chromosomal aberrations are often encountered when normal cells are exposed to mutagenic chemicals. In *in vivo* studies, only some tissues are amenable to chromosomal analysis, the prerequisite being that cells are capable of proliferation. This requirement limits the number and type of cells that can be studied, and is thus usually confined to bone marrow cells, blood lymphocytes, regenerating liver cells (Hittelman, 1990).

The types and frequencies of induced chromosomal aberrations depend on the mutagen used. The classical method to assess in *vivo* exposure to chemicals in laboratory animals is to study the frequency of chromosomal aberrations in bone marrow cells. Classical cytogenetic techniques can be used easily to detect

chromosome or chromatid aberrations (unstable aberrations), the frequency of which is a qualitative index of exposure to BNE.

Materials and methods

Materials

- Mouse bone marrow cells (BMCs)
- Human peripheral blood lymphocytes (HPBLs)

Reagents

- DL-Buthionine-S, R-sulphoximine (Sigma, USA): This is a potent inhibitor of the enzyme γ-glutamylcysteine synthetase of GSH pathway. BSO was freshly prepared in phosphate buffer solution (pH 7.4).
- 5-bromo-2-deoxyuridine powder (BUdR, Sigma, USA): This was used only for *in vitro* experiments. BudR was dissolved in RPMI 1640 culture medium and added to each culture tube at a concentration of 6 $\mu g\ ml^{-1}$.
- Phytohaemagglutinin M (Gibco, USA)
- RPMI 1640 with penicillin and streptomycin (Hyclone, USA)
- New born calf serum (Hyclone, USA)
- Bis-benzimide (Hoechst 33258, Sigma, USA) 50 μgml^{-1} working solution in double distilled water
- Colcemid (Gibco, USA)
- Giemsa, 3% (BDH Chemicals Ltd., UK)
- All other chemicals were of analytical grade.

Preparation of Aqueous extract of BNE

Aqueous extract of BNE was chosen since saliva is water based and in natural condition of human consumption the BNE components are extracted in it (Kumpawat and Chatterjee, 2003). Unprocessed, raw BNEs were purchased from the local market. After shelling the fibrous coats, 100g of BNE were ground and suspended separately in 125 ml of distilled water and mixed thoroughly to give a smooth paste. This was kept overnight at 4-8°C and the extract was collected by centrifugation. The extraction procedure was repeated once more by adding 125ml of water to the residue. Both the extracts were pooled which represents 100gm BNE in 250ml distilled water. The extracts were filtered through 0.22μm-porosity millipore filter and kept in liquid nitrogen for complete freezing. The filtrate was lyophilized in a Secfroid Lyolab BII lyophilizer. The lyophilized mass was kept at 4°C until use. The extract contained 4.23gm/100g water extractable material.

Treatment

Betel-Nut Extract (BNE): For *in vivo* study BNE (1mg/mouse/day) was administered orally in the drinking water. Oral administration was continued for 10, 30 and 45 days. In case of lime, it was administered orally through the drinking water and the pH was maintained at (~ 8.5-9.5). Lime and BNE treatment was also administered for 10, 30 and 45 days.

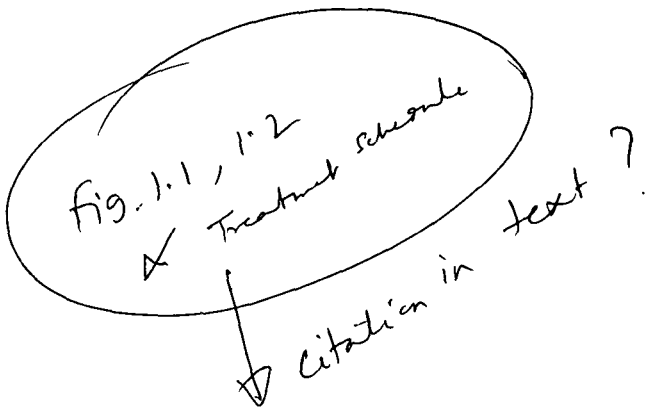
BSO: BSO was injected intraperitoneally in mice 10h before the first dose of BNE-extract administration. Thereafter for 10, 30, and 45 days treatment, subsequent treatment of BSO was given every 3rd day.

Lime: One group of mice were fed with BNE along with lime through drinking water, where the pH was adjusted to ~9.5 using lime (calcium hydroxide) which was obtained from the local market.

Preparation of metaphases

At the end of 10, 30 and 45 days treatment, mice were sacrificed by cervical dislocation with 3h prior treatment of colchicine (10mg kg^{-1} bw) in each animal. The femur bones were dissected out and the BMC were flushed out with 2ml of prewarmed (37°C) 0.075M KCl with the help of hypodermic syringe and a 26-guage needle. A single cell suspension was made in hypotonic solution and incubated for 15mts at 37°C. The cells were centrifuged at 1200rpm for 5min, and fixed in fixative, acetic acid: methanol, (1:3) for 30 minutes. The cells were washed in two changes of fixative, and finally resuspended in 1ml of the fixative and dropped onto a grease free chilled slide and flame dried.

In case of *in vitro* experiments, colcimid at a concentration of (0.01µgml^{-1}) was added 3h prior to harvesting. Hypotonic treatment was done for 20 min and cells were fixed and slides were prepared according to flame drying method as stated above.



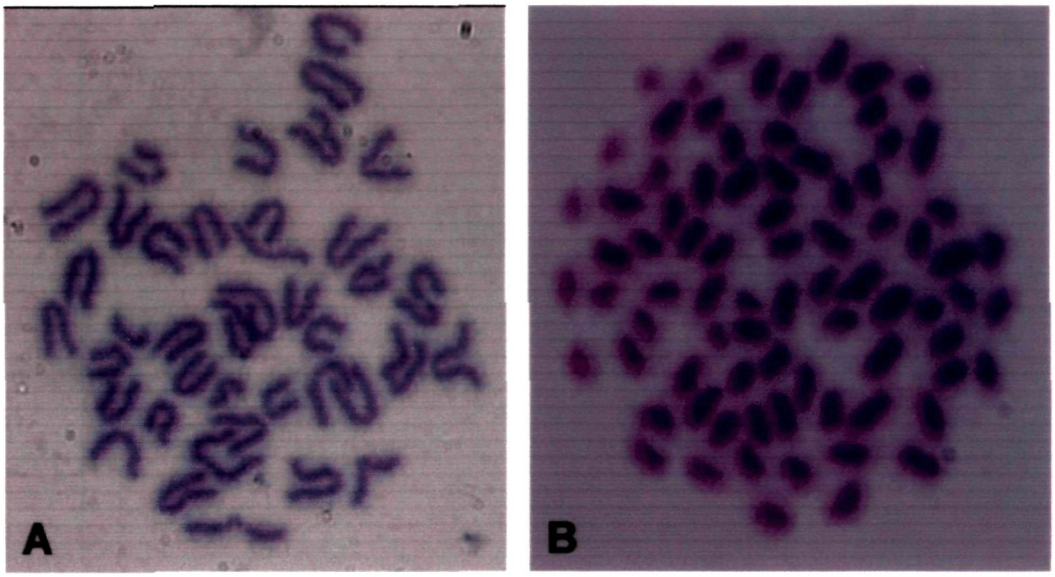


Figure 1.1 Microphotographs showing metaphase plates of mouse bone marrow cells
A. Normal chromosomes
B. Premature Anaphase Separation

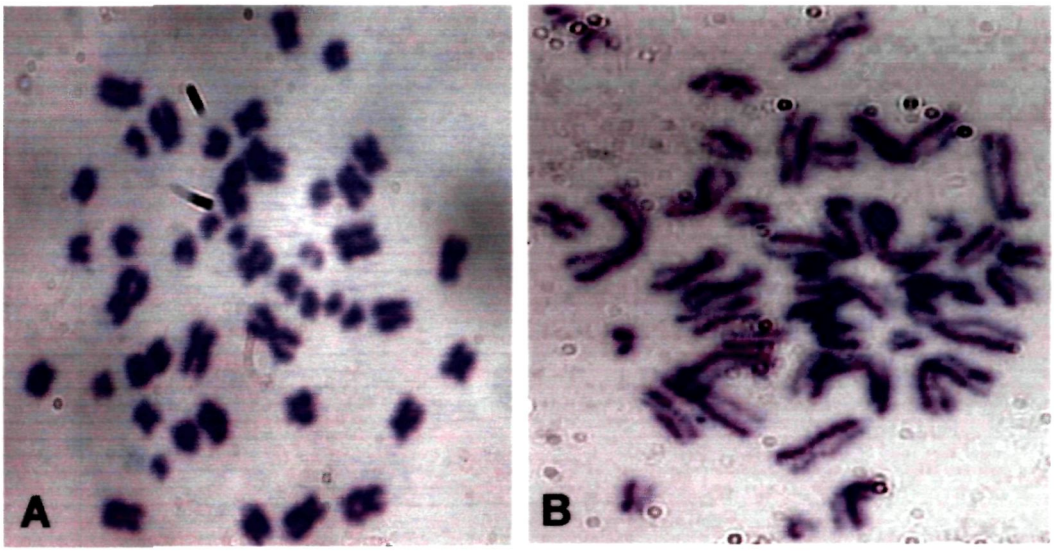
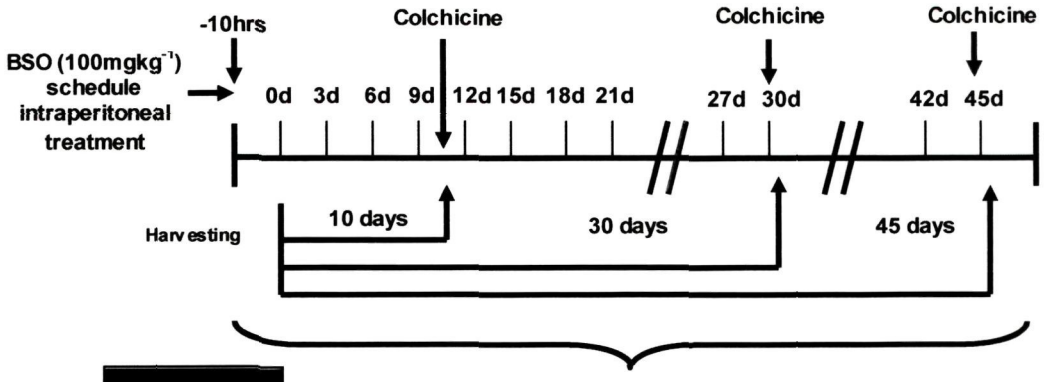


Figure 1.2 Microphotographs of normal metaphase plates of Human peripheral blood lymphocytes
A. Metaphase plate of 1st division cycle.
B. Metaphase plate of 2nd division cycle.

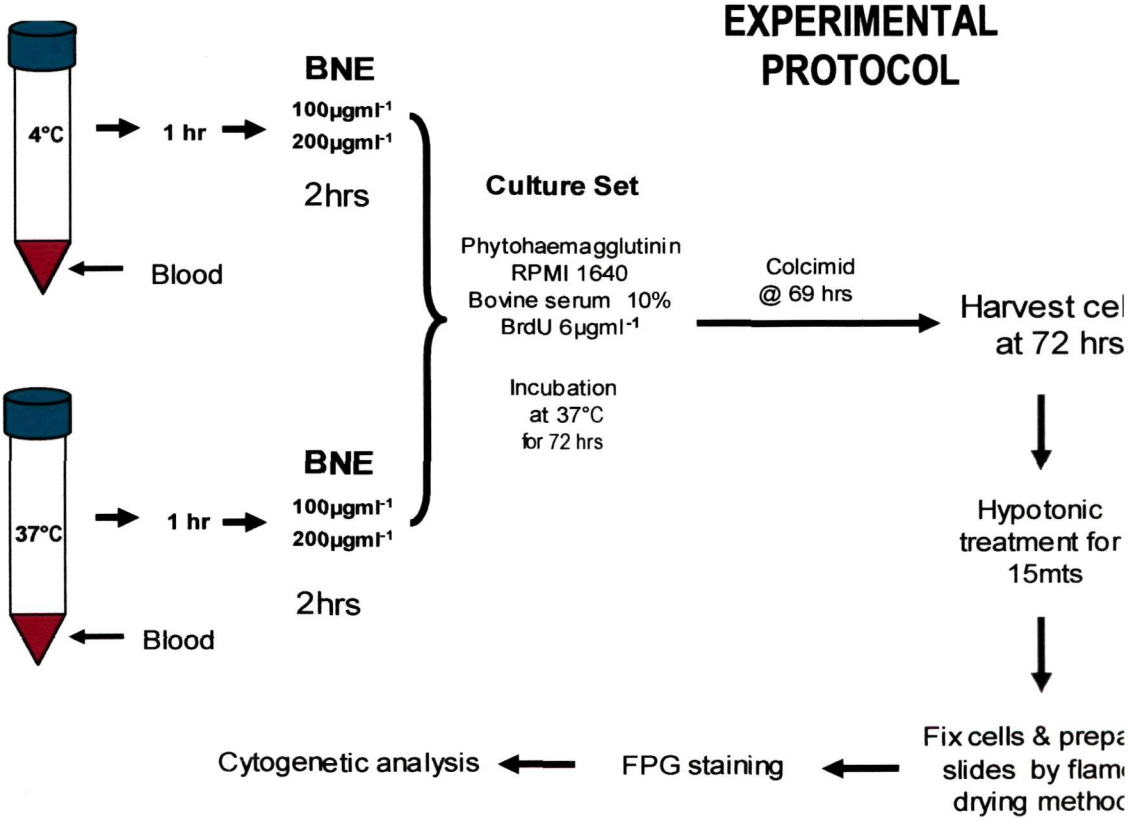
Treatment schedule for in vivo study



BNE and lime administered through drinking water

BNE 1mg/mouse/day
Lime ~ pH 9.5

EXPERIMENTAL PROTOCOL



Collection of human blood, its treatment and preparation of metaphases

Culture procedure

For all the *in vitro* experiments, blood was collected from healthy young male donors (25-30 years) by venipuncture with hypodermic syringe containing anticoagulant heparin. Heparinized blood was divided into two aliquots, one kept at 4°C for cold treatment and the other one at 37°C for one hour before BNE treatment was given. BNE was given at two doses, 100 and 200µg/ml. Two hours after BNE treatment culture was set up in medium containing RPMI 1640 with antibiotics, supplemented with 10% heat inactivated new born calf serum. For each culture 1ml blood was used. One set was kept at 4°C for an hour and the other set was kept at 37°C before the treatment was given and maintained at the same temperature during the treatment. In order to stimulate the G_0 lymphocytes 0.2ml of Phytohaemagglutinin M was added to each culture. To obtain differential staining, 6µgml^{-1} of 5-bromodeoxyuridine was added to each culture. All cultures were incubated at 37°C and were harvested at 72 h. Colcemid was added at a concentration of 0.01µg ml^{-1} during the last 3 h in all cultures. Hypotonic treatment was done for 18 min in (0.56%) KCl, and the cells were fixed in acetic acid and methanol (1:3) and slides were prepared. All experiments were repeated at least three times.

Differential staining for sister chromatids

For differential sister chromatid staining of prepared slides, the method of (Goto et al., 1975) was followed. The slides were treated for 15 min with Hoechst 33258 (50µg/ml) in dark, rinsed in distilled water and mounted in 2 x SSC (Saline sodium citrate, pH 6.8; Na-citrate 5.295gm + NaCl 10.519 gm + 600 ml double distilled water) and exposed to sunlight in moist condition for 25-40 min depending on the intensity of the sunlight. Slides were rinsed twice in distilled water and stained in 3 % Giemsa (BDH chemicals Ltd., England) for 3-4 min. They were rinsed in distilled water, air-dried and mounted in DPX.

Scoring and Statistical analysis

Slides were coded in random. In human lymphocytes CAs were scored from first cycle metaphases. Both in mouse BMC and in human lymphocytes CAs were scored mainly as chromatid breaks. For the studies of SCEs, metaphase cells with differentially stained chromatids (2^{nd} cycle metaphases) from each sample were scored.

For scoring cell cycle kinetics, metaphases were categorized in different division cycles based on their differential staining pattern. The cell cycle data were presented as average generation time (AGT) which is the ratio of BrdU duration (h) and replicative index (RI), where RI = (1xM1 + 2xM2 + 3xM3)/number of cells.

The statistical significance of the difference between the treated and untreated samples for mitotic index AGT and the frequency of SCEs was evaluated using the student's t-test. The statistical significance of the difference between treated and untreated samples for the frequency of aberrant metaphases and 1^{st} cycle metaphases was evaluated by the 2x2 contingency χ^2-test and for CAs a simple χ^2-test was used.

Results

The effect of BNE was studied both in vivo and in vitro mammalian systems. The *in vivo* study was conducted in mouse BMC and the *in vitro* study was done in human peripheral blood lymphocytes (HPBLs). Both the systems are well established and suitable for the assessment of cytogenetic effects. In both the systems BNE was found to induce delay in cell cycle and chromosome aberrations. The in vivo findings are shown in tables 1.1, 1.2 and 1.3, 1.4 and the in vitro findings are shown in tables 1.5 and 1.6.

IN VIVO:

Cell cycle kinetics:

BNE-induced delay in cell cycle (MI) in mouse BMCs were studied as positive controls to BSO+BNE, BNE + Lime treated samples and the data are presented in Table 1.1 and figure 1.3. The data indicate that BNE induced significant delay in cell cycle and the extent of delay was more in 30 days treated sample than 10 days. Although the basic cell cycle progression varied considerably among individual animals in each group, the induction of cell cycle delay by BNE is evident. The treatment of BNE in BSO-treated mice showed more delay than BNE alone and here 30 days treatment showed higher delay than 10 days. The delay was found to increase when BNE was given with lime and longer such treatment showed more significant delay than BNE alone.

Chromosomal aberration (CAs):

BNE-induced CAs in mouse BMCs were studied as positive controls to BSO+BNE, BNE + Lime treated samples and the data are presented in Table 1.2 and figure 1.4. CA's were mainly of the chromatid-break type which was induced by BNE. The frequency of chromatid break was increased marginally by BNE treatment for both 10 and 30 days with respect to untreated control. Treatment of BNE in BSO-treated mice increased the frequency of aberrations insignificantly. Moreover, treatment of BNE with lime also increased the frequency of chromatid breaks marginally. Interestingly when BNE and lime in combination treated to BSO-treated mice for 10 days it showed 13% chromatid breaks and as the treatment time was increased from 10 to 20, 30 and 45 days the

frequency of aberrations was reduced. In these studies premature sister-chromatid separation was observed in some cells that were treated with BNE and lime. The frequency of such premature anaphase separation was increased further if BNE and Lime treatment was given to BSO-treated mice. It is worth mentioning that such premature anaphase separation was found to occur in the presence of spindle inhibitor colchicines. Therefore, in order to confirm this observation a separate experiment was performed whose data are presented in Table 1.4. It was mentioned above that the pH of the drinking water with BNE was 9 to 9.5 after addition of lime. In untreated mouse this phenomenon was not observed, however, treatment with BNE alone induced such effect just about 1% even after 30 days of treatment. Interestingly, when lime combined with BNE the frequency of premature anaphase separation was increased. Such increment was significant when combined treatment of BNE and lime was given to BSO-treated mice. Here the frequency of premature anaphase separation was found to increase in a linear fashion. Table 1.4 shows that the frequency of premature anaphase separation was 3.8% after 10 days and over 15% at the end of 45 days of treatment. Figure 1.5 shows the picture of premature anaphase separation in mouse bone marrow chromosomes.

IN VITRO

The *in vivo* studies reveal that BNE causes delay in cell cycle and induce low frequency of chromatid aberrations. However, it is not clear whether initial damages on DNA induced by BNE are more or not. Therefore, in vitro study was conducted in HPBLs to assess the initial damage induced by BNE. To address this problem we have pretreated the cells at 4°C for 1hour before the BNE treatment was given. The rationale for BNE-treatment at 4°C is based on the premise that repair machinery can not function at 4°C. After treatment at 4°C the culture was set up at 37°C. With another set the treatment was given at 37°C and then culture was set up.

Cell cycle kinetics

BNE-induced delay on cell cycle at 37°C and 4°C are shown in table 1.5 and figure 1.7. The induction of delay on cell cycle by BNE was clearly dose dependent. At 100 $\mu g\ ml^{-1}$

of BNE the frequency of M1 cells was 53% and mitotic index (MI) was 4.13 whereas with 200 μg ml^{-1} of BNE treatment the frequency of M1 was 68% and the MI was 3.71. Such delay in cell cycle was increased significantly while BNE treatment was given at 4°C.

Chromosomal aberrations (CAs)

Table 1.6 and figure 1.6 reveals the aberration status in human PBLs treated with BNE both at 37°C and 4°C. The data shows that BNE induced only chromatid breaks in a dose dependant manner. The frequency of chromatid breaks as well as aberrant metaphases was increased while the BNE treatment was given at 4°C than 37°C.

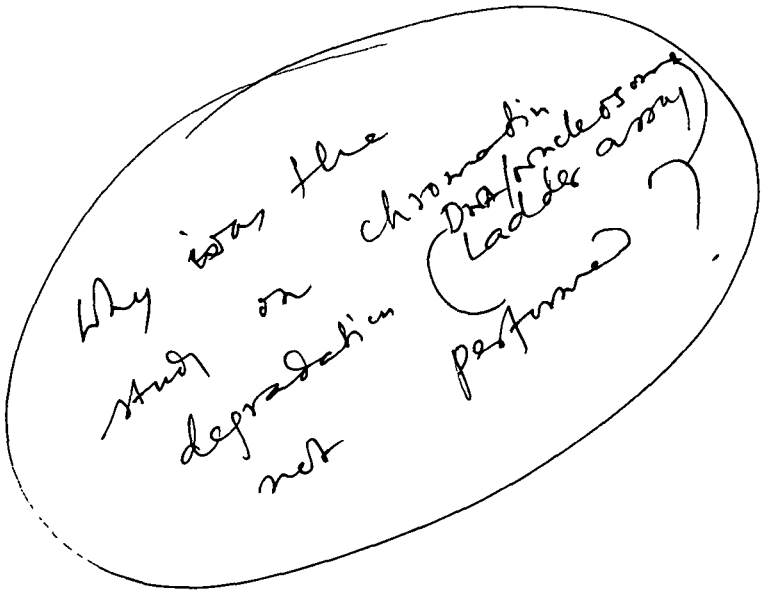


Table 1.1 Induction of cell cycle delay in mouse BMC's by BNE, BSO, and Lime

Experimental condition	Treatment days	Mitotic Index TM/Total cells	Mitotic Index %	Mean ± SEM	Range
Untreated	-	084/1018	07.73		
		139/1169	11.89		
		129/1116	11.55		
		107/1070	10.00		
		094/1018	09.23	**10.08 ± 0.76**	7.73 - 11.89
BNE	10	226/3372	06.70		
		176/2882	06.10		
		244/3275	07.45		
		083/1560	05.32		
		146/2622	05.56		
		312/4379	07.12		
		158/2314	06.83		
		132/2356	05.60	**6.33 ± 0.28**	5.32 - 07.45
	30	155/3276	04.73		
		125/2831	04.41		
		166/3342	04.96		
		111/2219	05.00		
		123/3154	03.89		
		103/2095	04.91		
		042/1086	03.89		
		044/1164	03.78	**4.45 ±0.18**[#]	3.78 – 5.00
BSO + BNE	10	098/2316	04.23		
		023/0750	03.06		
		044/1334	03.20		
		073/2040	03.57		
		049/1547	03.16	**3.41 ± .022**[#]	3.16 – 4.23
	30	085/2516	03.31		
		043/2300	01.86		
		028/1238	02.26		
		030/1436	02.08		
		032/1203	02.66	**2.43 ± 0.25**	1.86 - 3.31
BNE + Lime	10	086/1753	04.90		
		068/1156	05.88		
		168/3271	05.13		
		060/1146	05.23		
		050/1319	03.79		
		099/2658	03.27	**4.70 ± 0.39**[#]	3.27 -5.88

	30	082/1938	04.13		
		061/2295	02.65		
		030/1055	02.84		
		051/2122	02.49		
		048/1988	02.46		
		030/1255	02.84		
		051/2427	02.10		
		048/1782	02.69	**2.77 ± 0.21**	2.40 - 4.13
BSO +	10	047/1596	02.94		
BNE +		079/2368	03.33		
Lime		**103/2121**	**04.88**		
		061/1361	04.48		
		050/1223	04.08		
		056/2127	02.63		
		045/1198	03.52		
		072/2304	03.13		
		074/2342	03.16		
		111/3144	03.53		
		034/1114	03.05		
		026/ 1054	02.46		
		041/1069	03.83		
		035/1134	03.08		
		060/1440	04.17	**3.48 ± 0.18**	2.46 - 4.88
	30	047/1328	03.53		
		046/1398	03.29		
		056/1397	04.01		
		049/1353	03.62		
		043/1149	03.74	**3.63 ± 0.11**	3.29 - 4.01
	45	049/1325	03.69		
		041/1171	03.50		
		049/1227	03.99		
		053/1423	03.72		
		040/1102	03.63	**3.71 ± 0.08**	3.50 - .36

BNE: 1mg/mouse/day **BSO**: 100mg/kg^{-1} **Lime**: pH: 8.5 – 9.0

$^{\#}$ $p<0.05$; student's t-test compared with respective treatments

Table 1.2

Chromosomal aberration in mouse BMC's induced by BNE, BSO and Lime

Experimental Condition	Treatment days	TM	Ab.M (%)	Mean ± SEM	Aberration % Chd.Bk	Aberration % X ± SEM
Untreated	-	175	0		0	
		188	01		01	
		265	02		02	
		102	02		02	
		198	01	**1 ± 0.8**	01	**1 ± 0.4**
BNE	10	168	03		03	
		127	03		03	
		178	03		03	
		150	03		03	
		272	03		03	
		255	04		04	
		214	03		03	
		144	02	**3 ± 0.18**	02	**3 ± 0.2**
	30	171	05		05	
		163	08		10	
		198	08		08	
		175	03		03	
		206	01		01	
		154	03		03	
		146	04		04	
		183	04	**5 ± 0.9**	04	**5 ± 1.1**
BSO + BNE	10	162	05		05	
		056	14		14	
		121	08		08	
		138	06		06	
		120	05	**8 ±1.7***	05	**8 ±1.7**
	30	169	08		08	
		156	08		08	
		116	12		16	
		124	10		13	
		106	09	**9±0.7**	09	**11 ±1.6**
BNE + Lime	10	136	05		05	
		177	10		10	
		053	10		09	
		068	10		10	
		124	04		04	
		162	11	**8±1.2***	12	**8 ±1.3**

	30	201	06		06	
		182	05		05	
		202	08		08	
		171	07		08	
		193	07		08	
		202	08		10	
		171	07		08	
		193	07	**7 ± 0.4**	08	**8 ± 0.6**
BSO +	10	226	12		12	
BNE +		233	11		11	
Lime		185	13		13	
		140	16		16	
		141	08		08	
		337	12		14	
		160	13		13	
		293	17		17	
		373	15		16	
		344	12		12	
		176	09		09	
		155	10		10	
		166	14		14	
		198	19		19	
		229	11	**13 ± 0.8***	11	**13 ± 0.9**
	30	257	03		04	
		299	04		05	
		326	03		04	
		312	04		05	
		344	03	**3 ± 0.2**	04	**4 ± 0.2**
	45	202	02		02	
		170	02		02	
		314	03		03	
		265	02		02	
		270	02	**2 ± 0.2**	02	**2 ± 0.2**

BNE: 1mg/mouse/day **BSO**: 100mg/kg^{-1} **Lime**: pH: 8.5 – 9.0

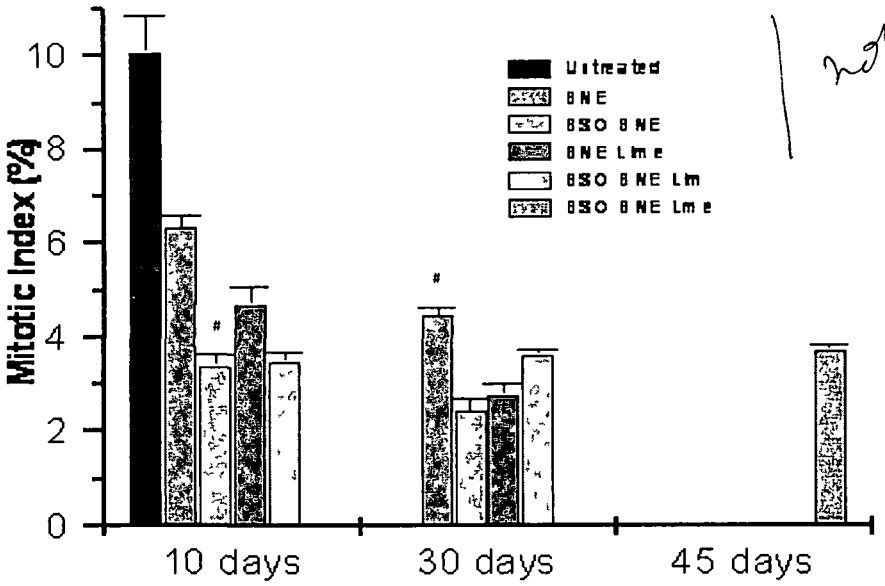
TM: Total metaphase **Abt.M:** Aberrant metaphase **Ch.bk:** Chromatid break

*$p<0.01$; 2x2 contingency X^2-test compared to respective controls

Table 1.3
Pooled data on the induction of CA's and delay in cell cycle kinetics by BNE, BSO and Lime in mouse BMC.

Experimental Condition	Treatment Days	TM	Ab.M (%) ± SEM	Chd.bk (%) ± SEM	Mitotic Index TM / Total cells	Mitotic Index (%) ± SEM	No of mice
Untreated	-	928	1±0.8	1± 0.4	553 / 5391	10.1 ± 0.8	5
BNE	10	1508	3±0.2	3± 0.2	1477 / 22760	6.5 ± 0.4	8
	30	1396	5±0.9	5± 0.9	869 / 19149	4.4 ± 0.2#	8
BSO+BNE	10	597	8±1.7*	8±1.7*	287 / 7987	3.4 ± 0.2#	5
	30	671	9±0.7	11±1.6	218 / 8693	2.4 ± 0.35	
BNE+Lime	10	720	8±1.2*	8±1.3*	531 / 11303	4.7 ± 0.4#	6
	30	1515	7±0.4	8±0.6	401 / 14862	2.7 ± 0.2	8
BSO+BNE+	10	3356	13±0.8*	13±0.8*	894 / 19510	3.5 ± 0.2	15
Lime	20	1194	5±0.7	6±0.5	176 / 5976	2.9 ± 0.1	5
	30	1538	3±0.2	4±0.2	241 / 6625	3.6 ± 0.1	5
	45	1221	2±0.2	2±0.2	232 / 6248	3.7 ± 0.1	5

BNE: 1mg/mouse/day **BSO**: 100mg/kg^{-1} Lime: pH: 8.5 – 9.0
TM: Total metaphase **Abt.M:** Aberrant metaphase **Ch.bk:** Chromatid break
*$p<0.01$; 2x2 contingency X^2-test compared to respective controls
#$p<0.05$; student's t-test compared with respective treatments



not clear !

Figure 1.3 Effect of BNE with or without lime on cell cycle kinetics in mouse BMC's. Each point and bar represents a mean ± SEM value

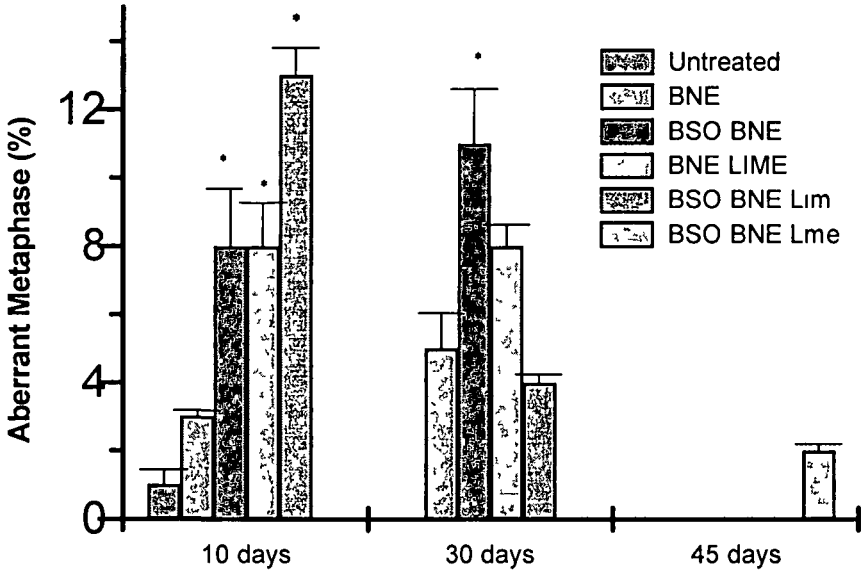


Figure 1.4 Effect of BNE with or without lime on the frequency of aberrant metaphases in mouse BMC's. Each point and bar represents a mean ± SEM value.

Table 1.4

Premature Anaphase Separation in mouse BMC's induced by BNE, BSO and Lime

Experimental Condition.	Treatment Days	TM	Premature Anaphase Separation No.	(%)	Mean ± SEM
Untreated	--	284	0	0	
		139	0	0	
		129	0	0	
		107	0	0	
		194	0	0	**0**
BNE	30	146	1	0.68	
		183	3	1.63	
		197	2	1.01	
		346	2	0.57	
		254	1	0.39	**0.85 ± 0.2**
BNE + Lime	30	202	07	3.46	
		171	10	5.84	
		193	12	6.21	
		221	11	4.98	
		184	06	3.24	**4.76 ± 0.6#**
BSO + BNE + Lime	10	176	10	5.68	
		155	07	4.51	
		166	06	3.61	
		198	06	3.03	
		229	05	2.18	**3.80 ± 0.6**
	20	238	08	3.36	
		191	14	7.34	
		146	11	7.53	
		248	15	6.05	
		172	13	7.56	**6.07 ± 0.9**
	30	257	29	11.28	
		299	27	09.03	
		294	32	10.89	
		326	42	12.88	
		312	41	13.14	**11.44 ± 0.1#**
	45	202	35	17.32	
		170	29	17.05	
		270	37	13.70	
		252	39	15.48	
		376	49	13.03	**15.32 ± 0.8#**

BNE: 1mg/mouse/day **BSO**: 100mg/kg^{-1} **Lime**: pH: 8.5 – 9.0 TM: Total metaphase
BNE: Betel-nut Extract, **BSO:** Buthionine sulfoximine, **Abt.M:** Aberrant metaphase, **Ch.bk:** Chromatid break
#$p<0.05$; student's t-test compared with respective controls

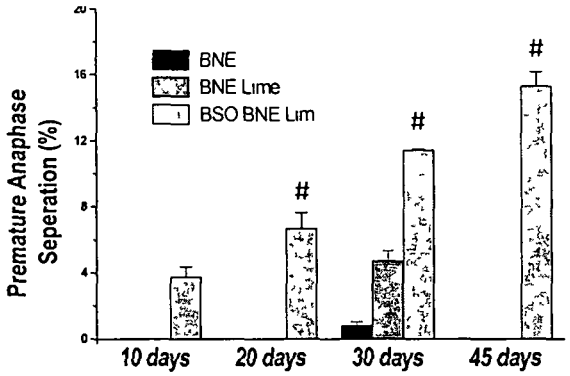


Figure 1.5 Effect of BNE with or without lime and BSO on the frequency of premature anaphase separation in mouse BMC's Each point and bar represents a mean ± SEM value.

Table 1.5
Induction of delay in cell kinetics by BNE in HPBLs in vitro

#	Treatment condition		TM	%		AGT	Mitotic Index	
				M1	M2		TM2/ Total cells	(%)
1	Untreated	4°C	157	54	31	44.7	53/1295	4.09
2			219	57	29	45.8	82/1760	4.66
3			124	52	25	41.9	48/1230	3.90
				54.3±1.5		**44.2±1.2**		**4.21±0.2**
1		37°C	198	48	33	42.0	93/1720	5.41
2			182	54	29	44.2	60/1133	5.29
3			194	56	24	43.9	77/1503	5.12
				52.7±2.4		**43.3±0.7**		**5.27±0.1**
1	BNE100μgml^{-1}	4°C	153	71	24	52.9	37/1143	3.23
2			238	72	19	52.9	43/1291	3.33
3			192	63	28	51.8	45/1509	2.83
				68.7±2.8		**52.5±0.4**		**3.13±0.2**
1		37°C	177	69	21	65.4	67/1318	4.85
2			208	60	27	47.1	43/1291	3 33
3			127	58	31	48.6	33/1120	2.95
				62.3±3.4		**53.7±5.9**		**3.71±0.6**
1	BNE200μgml^{-1}	4°C	123	87	11	62.6	26/1087	2.39
2			115	87	10	64.8	43/1405	3.06
3			110	74	23	55.0	29/1213	2.39
				82.7±4.3*		**60.8±2.9**		**2.61±0.2#**
1		37°C	206	67	25	51.0	55/1127	4.85
2			130	66	25	51.4	59/1186	4.97
3			117	70	19	51.1	32/1245	2.57
				67.7±1.2		**51.6±0.2**		**4.13±0.8**

AGT: Average Generation Time TM: Total metaphase
*$p<0.01$; 2x2 contingency X^2-test compared to respective controls
#$p<0.05$, student's t-test compared with respective treatments

Table 1.6

Chromosomal aberration in HPBL's with BNE with Cold treatment

#	Treatment condition		TM	Abt.M (%)	Ch.bk (%)
1	Untreated	4°C	67	1	2
2			92	3	3
3			118	1	1
				1.7±0.6	**2.0±0.5**
1		37°C	52	1	2
2			70	2	2
3			124	1	1
				1.3±0.3	**1.6±0.3**
1	BNE 100 (μg ml^{-1})	4°C	78	10	10
2			110	12	26
3			63	25	25
				15.7±4.7	**20.3±5.2**
1		37°C	68	7	7
2			36	9	14
3			74	19	21
				11.7±3.7	**14±4.1**
1	BNE 200 (μg ml^{-1})	4°C	47	25	25
2			87	19	28
3			94	35	37
				26.3±4.7*	**30±3.6***
1		37°C	47	25	35
2			55	10	13
3			83	21	22
				18.7±4.5	**20±3.6**

TM: Total metaphase **Abt.M:** Aberrant metaphase **Ch.bk:** Chromatid break
*$p<0.01$; 2x2 contingency X^2-test compared to respective controls

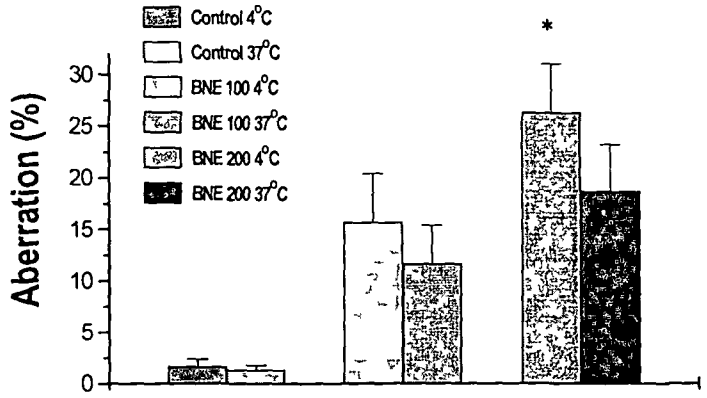


Figure 1.6 Effect of BNE with or without lime on the frequency of aberrant metaphases in human HPBL's. Each point and bar represents a mean ± SEM value.

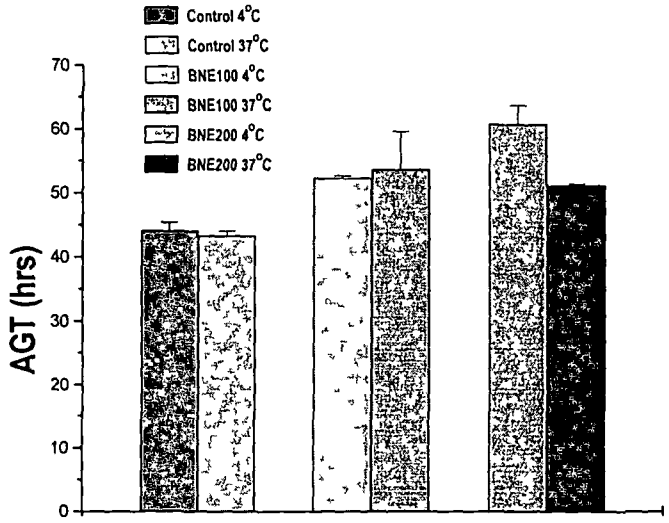


Figure 1.7 Effect of BNE with or without lime on cell cycle kinetics in human HPBL's. Each point and bar represents a mean ± SEM value

Discussion

Betel quid chewing is a common habit in oriental countries. Extensive epidemiological studies show that the high incidence of oral and esophageal cancers in these countries are associated with betel quid chewing (Jussawalla and Deshpande 1971; Hirayama 1979; Sanghvi 1981). Mutagenecity and genotoxicity of betel alkaloids, polyphenol and tannin fractions have been reported (Deb and Chatterjee 1998; Jeng et al., 2001). It was also demonstrated that the raw betel nut extract of Meghalaya can induce DNA damage and such damage is increased when endogenous GSH is depleted by buthionine sulfoximine (BSO) (Kumpawat and Chatterjee 2003; Kumpawat et al., 2003). It is true that the amount of DNA damage induced by RBNE was low in both lymphocytes and mouse BMC and it was increased marginally in the mouse BMC even after 30 days of treatment (Kumpawat et al., 2003). Therefore, the purpose of this study was to address the following two questions:

1. whether the presence of lime along with raw-BNE induces more DNA lesions than BNE alone?
2. whether raw-BNE induces higher DNA lesions initially which later repaired or it induces extremely low level of DNA damage?

It was observed that there was an increase in the frequency of CA in mouse BMCs treated with both lime and BNE. The use of lime by betel-quid chewers increases the pH which plays a crucial role in the genesis of oral cancer, particularly in areas of the world where no tobacco is used in the quid. A number of studies have demonstrated that pH, as determined by the free $Ca(OH)_2$ content of lime, is the major determinant of the generation of reactive oxygen species from betel quid components (Nair et al., 1987, Nair 1990). It was noted that hydrogen peroxide (H_2O_2) and superoxide radicals are produced during autoxidation of BNE-polyphenols when the pH level is greater than 9.5. In fact it was not clear whether BNE-components could generate ROS, however, there are reports indicating the generation of ROS from BNE ingredients under alkaline conditions (Nair et al., 1987). It was shown that post treatment of superoxide dismutase (SOD) could protect chromosome aberrations induced by arecoline indicating the involvement of superoxide radicals in the induction of CA by

ARC (Kumpawat et al., 2003). Another important component of BNE and betel leaf is the transition metal ions such as Cu^{2+}, Mn^{++}, Fe^{2+} and Fe^{3+}, promoting the production of ROS by BNE. Since ROS are important in the initiation and promotion of cancer therefore, it seems that all the components generating from the kwai mixture have the potentialities of cancer induction. It has also been suggested that the metabolic activation may produce a variety of BNE-specific nitrosamines which could be the primary cause of oral mucosal lesions (Hoffmann et al., 1994).

From the previous reports it has been established that BNE treatment depletes GSH levels significantly to 51% and 37% after 1 day and 5 day treatment in mouse BMCs (Kumpawat and Chatterjee 2003). Biochemical studies have found that ARC and arecaidine react with thiol groups both in vivo and in vitro to produce cysteine 3-alkylation adducts. This may explain the cause of the depletion of GSH in oral fibroblasts and keratinocytes and buccal mucosa of betel-quid chewers and may render the cells susceptible to potential further attack by other BNE components or environmental toxicants. Moreover, further depletion of GSH by BSO increased the frequency of CA induced by both BNE and lime.

Our study shows that BNE mainly induced chromatid breaks and this induction can be modulated considerably when mice were treated with BSO. In this study BNE failed to induce any exchanges. This indicates that the DNA lesions induced by BNE are not appearing at the same time or close proximity and therefore that they failed to associate to form exchanges. One of the goals of the present study was to investigate the extent of the initial damage or lesions in cells treated with BNE. The results reported can be interpreted in the light that DNA repair enzymes are rapidly activated during the cellular response to DNA damage and are part of a large network of safeguard mechanisms protecting cells from genotoxic damage (Enoch et al., 1995). It seems that BNE induced damage can possibly have a higher baseline of damage, which are not seen in the cells fixed after 52 or 72h of treatment possibly due to the normal repair mechanisms that work in the system. We have attempted to evaluate the extent of DNA damage (initial damage) by inhibiting the normal repair process in lymphocytes by treating the cells at 4°C. The data revealed that there was a marginal increase in the frequency of chromatid breaks at 4°C than at 37°C and accordingly the

delay in cell cycle kinetics was higher at 4°C than at 37°C. Therefore, it is clear that BNE does not induce initial DNA lesions substantially higher in frequency.

In the present study as well as earlier studies showed that raw BNE induced a significant delay in the cell cycle in both systems and depletion of endogenous GSH by BSO increased such BNE-induced delay in mouse BMC. It has been reported that BNE can reduce the clonal cell growth of buccal epithelial cells and gingival keratinocytes (Sundqvist et al., 1991). Earlier it was demonstrated that ARC induced delay in cell kinetics in mouse BMC (Deb and Chatterjee 1998) and a similar observation was made by others in human gingival fibroblasts (Van Wyk et al., 1994) and also in skin fibroblasts (Jeng et al., 2001). It is known that ARC, a component of raw BNE, by losing only one of its methyl groups may bind with nucleic acid and protein (Boyland and Nery 1969) and by doing so it could induce delay in cell kinetics. However, the present and earlier data indicate that raw BNE does not induce substantial DNA damages and therefore the induction of delay in cell cycle may not be primarily due to DNA damages in the cells. It was shown that aqueous extract of betel nut alone depleted endogenous GSH in mouse BMCs, but not in human lymphocytes (Kumapawat et al., 2003) and this could also be responsible for the delay since cellular GSH is important for cell proliferation (Van Wyk et al., 1994). Although in the above mentioned study (Kumpawat et al., 2003) the aqueous extract of betel nut alone did not deplete endogenous GSH level but the lymphocytes of heavy chewers of raw betel-nut showed significant level of depletion of endogenous GSH with respect to non-chewers (Kumpawat and Chatterjee 2003). However, the induction of delay in cell cycle by raw BNE observed in lymphocytes in the present study could be dependent on multivariate factors.

A novel aspect in the present study is the treatment of BNE and lime to the mouse BMCs which showed an induction of premature anaphase separation besides chromatid breaks. The frequency of premature anaphase separation was 5% after 30 days of BNE treatment. This frequency was enhanced significantly when BNE and lime was treated to BSO mediated GSH-depleted cells. The frequency was increased to 11% after 30 days and 15% after 45 days. Sister chromatids that have separated prematurely are a hallmark of a defective mitotic checkpoint and have been observed

in yeast *mad2* mutants and *Drosophila bub1* mutants (Basu et al., 1999, Alexandru et al., 1999). The presence of such precocious anaphase in checkpoint-deficient Hct-116 and in mouse embryonic fibroblasts was demonstrated (Michael et al., 2001). Defects in mitotic checkpoint have been correlated with chromosome instability (CIN) in human cancer cells but have not yet been shown to be a direct cause of the CIN phenotype (Cadhill et al., 1998). Genetic instability in cancers occurs frequently with whole chromosomes, referred to as CIN. CIN cells become aneuploid, a hallmark of cancer that is associated with aggressive tumor behavior and a poor prognosis (De Vita et al., 1997). Aneuploidy may facilitate tumorigenesis or tumor progression through the loss of tumor-suppressor gene function. Therefore, the present observation of premature anaphase separation could lead to develop aneuploid cells since deletion of one MAD2 allele show premature sister-chromatid separation in the presence of spindle inhibitors and an elevated rate of chromosome mis-segregation events in the absence of these agents (Michael et al., 2001).

The present study shows that the induction of premature anaphase separation was more in GSH-depleted cells exposed to BNE and lime. This indicates that the long term exposure of raw BNE with lime depletes the endogenous GSH level in buccal epithelial cells and may render the cells more susceptible to further attack and induce premature anaphase separations which ultimately lead to develop aneuploid cells. It is worth mentioning that the treatment of BNE and lime to BSO-treated mouse BMCs showed gradual decrease in the frequency of chromatid aberrations as the treatment period was increased. This could be attributed due to increased induction of cells with premature anaphase separation which in turn reduce the frequency of damaged cells with chromatid aberrations.

Mutagens and carcinogens of foods and beverages could conceivably undergo their first changes in the oral cavity. There they could interact each other as well as with saliva-borne compounds, leading to enhanced or reduced mutagenic and carcinogenic activities. In the case of endogenous nitrosamine formation, the importance of nitrate and nitrite-carrying saliva has already been recognized (Walters et al., 1979). Of particular concern should be the situations where the mucosa of oral cavity, pharynx and esophagus are exposed for prolonged periods to mutagens and/or carcinogens

which become released into the saliva during habitual chewing. Habitual chewers of the tribal population of the North-Eastern region of India chew raw betel-nut (hence more ARC), lime and betel-leaf 15 to 20 times a day for 15-30 min each time and hence the duration of exposure in the mouths of humans is many times longer than that expected in our experimental systems. From the present study, it seems that the long term exposure of raw BNE with lime depletes the endogenous GSH level in buccal epithelial cells and may render the cells more susceptible to further attack by ROS. During this process there is likely possibility of damaging one of the mitotic check-point genes which ultimately induce premature anaphase separation and that could lead to develop aneuploid cells. However, further study is needed to see that raw BNE induced oral and esophageal cancer has a defect in the mitotic checkpoint genes.

CHAPTER II

LOSS OF HETEROZYGOSITY STUDIES ON CHROMOSOME 9p21

Introduction

The past three decades have seen an unrelenting quest to understand the reproduction of normal and cancerous cells. Cells in early embryo can proceed through continuous cycles of DNA replication and nuclear division at astonishing speed. DNA replication starts as soon as mitosis ends and a full cycle of cell division are completed in a mere half-hour. But as embryogenesis unfolds and the demands of cell life in a complex environment set in, a bureaucracy arises. The biology of cell division and differentiation, is exceedingly similar in both normal and cancer cells. The cancer cell differs from its normal counterpart in that it is aberrantly regulated. Cancer cells generally contain the full complement of biomolecules that are necessary for survival, proliferation, differentiation, cell death, and expression of many cell-type- specific functions. Failure to regulate these functions properly, however, results in an altered phenotype and cancer. Four cellular functions tend to be inappropriately regulated in a neoplasm. First, the normal constraints on cellular proliferation are ineffective. Second, the differentiation program can be distorted. The tumor cells may be blocked at a particular stage of differentiation, or they may differentiate into an inappropriate or abnormal cell type. Third, chromosomal and genetic organization may be destabilized such that variant cells arise with high frequency. Some variants may have increased motility or enzyme production that permits invasion and metastases. Finally, the tightly regulated cell death program (apoptosis) may be dysregulated. Cancer is a group of diseases in which cells grow and spread unrestrained throughout the body. Cancers can arise in nearly any type of cell which retains the ability to divide. Although there are more than 100 forms of cancer, the basic processes underlying all of them are very similar. The process by which normal cells become cancerous is called carcinogenesis. Two gene classes play major roles in choreographing the cellular life cycle: proto-oncogenes initiate cell growth and division, and tumor suppressor genes inhibit cell growth and division. When proto-oncogenes go awry, and become oncogenes, they maintain continuous growth signals. For a tumor to develop, mutations usually must occur in several genes. To comprehend the biology of cancer, it is necessary to understand how these functions are controlled in normal cells and how they become uncontrolled in cancer cells. The majority of human tumors arise

from epithelial tissues. Epithelia are sheets of cells that line the walls of cavities and channels or, in the case of skin, serve as the outside covering of the body. By the first decade of the twentieth century, detailed histological analyses had revealed that normal tissues containing epithelia are all structured similarly. Thus, beneath the epithelial cell layers in each of these tissues lies a basement membrane (sometimes called a basal lamina); it separates the epithelial cells from the underlying layer of supporting connective tissue cells, termed the stroma. The basement membrane is a specialized type of extracellular matrix (ECM) and is assembled from proteins secreted largely by the epithelial cells. In all cases, these basement membranes serve as a structural scaffolding of the tissue.

Cancers that arise from cells that cover external or internal body surfaces are carcinomas. The most common examples of these are lung, breast and colon cancer. Sarcomas are cancers that arise from cells in supporting tissues such as bone, cartilage, fat, connective tissue and muscle. Lymphomas arise in the lymph nodes and tissues of the immune system and leukemias are cancers of the immature blood cells that develop in the bone marrow. An increase in the number of dividing cells creates a growing mass of tissue called a tumor or neoplasm. There are several stages to cancer development. Hyperplasia is tissue growth due to an excessive cell division rate but cell structure and the arrangement of cells is normal. Hyperplasia can be due to an irritating stimulus. Dysplasia is excessive cell proliferation along with the loss of normal tissue arrangement and normal cell structure to some degree. Sever dysplasia is referred to as a carcinoma in situ and is uncontrolled cell growth. Epithelia are of special interest here, because they spawn the most common human cancers the carcinomas. These tumors are responsible for more than 80% of the cancer-related deaths in the western world. Included among the carcinomas are tumors arising from the epithelial cell layers of the gastrointestinal tract which includes mouth, esophagus, stomach, and small and large intestines as well as the skin, mammary gland, pancreas, lung, liver, ovary, gallbladder, and urinary bladder. Tumors that arise from epithelial cells forming these protective cell layers are said to be squamous cell carcinomas. Squamous cell carcinoma of the head and neck is an epithelial malignant disease arising from the mucosa of the aerodigestive tract (oral cavity, larynx, oropharynx,

hypopharynx and esophagus). According to which prolonged exposure of the epithelium to certain carcinogenic agents (Betel nut, tobacco and alcohol) could induce cellular alterations and tumor formation at multiple anatomical sites. There have been few data showing evidence of inheritance in head and neck squamous cell carcinoma (HNSCC) (Ankathil et al., 1996).

Both environmental and endogenous factors can interact at various steps in tumor development and progression. In most cancers, environmental factors are involved in carcinogenesis, but there is also clear evidence for genetic factors being involved in cancer susceptibility (Strachan and Read, 1999). Hereditary cancer cases are estimated to comprise approximately 1–5% of all cancers (Arver et al., 2000; Cornelisse and Devilee, 1997; de la Chapelle and Peltomaki, 1995; Gertig and Hunter, 1998; Lindblom and Nordenskjold, 2000). The majority of cancers are a consequence of external factors that act in conjunction with both genetic and acquired susceptibility. Several environmental exposures (such as tobacco smoke, occupational exposure, air pollution and diet) have clearly been associated with many cancers. Of all the environmental causes, tobacco smoking is the most important single risk factor, causing many types of cancers, in particular, lung cancer. Though only small minorities of all cancer cases are caused by an inherited gene mutation, molecular studies of these cases have been of great help in increasing the general knowledge of the basic mechanisms underlying cancer development (Cornelisse and Devilee, 1997). As the penetrance of the disease is known to be influenced by a number of factors, such as environmental exposures, the estimation of the shared role of genetic and environmental factors in the development of cancer is very difficult (Fearon, 1997).

Tumor suppressor genes

Tumor suppressor gene products normally inhibit cell proliferation by controlling cell cycle progression or directing damaged cells into apoptosis. Thus, these genes restrict the tumourigenic and metastatic processes (Levine, 1993). According to the classical Knudson's "two hit" model of tumourigenesis, an inactivation of both copies of a tumor suppressor gene leading to loss of function is required for the development of a neoplastic cell (Knudson, 1971). In hereditary diseases, the first hit in the germ line cells represents a mutated copy of a tumor suppressor gene and the second hit is a

somatic inactivation of the other allele. In cases of sporadic tumors, it has been shown that there are two somatic inactivating events that affect both copies of the tumor suppressor gene. The inactivation may happen through mutations or through epigenetic modifications (e.g., methylation) (Sugimura and Ushijima, 2000). Knudson's hypothesis that two hits are required for the full inactivation of a tumor suppressor gene has been shown to be correct in any human cancers (Jones and Laird, 1999). However, as our knowledge grows about genes involved in cancer, the traditional definition of tumor suppressor genes may need to be broadened (Haber and Harlow 1997; Le Beau et al., 1998). Mutations in tumor suppressor genes may be hereditary and thus present in the germline of individuals leading to genetic susceptibility to cancer, or they may be somatic when two individual inactivating events occur at the same locus (Haber and Harlow, 1997).

Oncogenes

Oncogenes are mutated forms of proto-oncogenes. The normal function of many of these genes is to control the cell cycle by stimulating cell proliferation. Activating gain of- function mutations of proto-oncogenes may cause malignant transformation (Cornelisse and Devilee, 1997; Karp et al., 1996). Abnormal activation can occur in many different ways, by chromosomal translocation resulting in gene combination, by point mutations resulting in an altered protein product, or by amplification resulting in overexpression of the oncoprotein which again results in excessive cell proliferation (Cornelisse and Devilee, 1997; Munger, 2002). Although oncogenes are involved in many different types of cancer, they rarely seem to play a central role in hereditary cancer.

Loss of Heterozygosity (LOH)

There are 22 pairs of homologous chromosomes in each human cell in males, 23 pairs in females. Except for those on the X and Y chromosomes in males, each gene in our genome is present in two copies, one on each of two homologous chromosomes. These two copies or alleles are usually slightly different from one another as a result of random mutations over evolutionary times. Should one allele become mutant, the cell is said to be heterozygous at this locus (gene). An organism is a heterozygote or is heterozygous at a locus or gene when it has different alleles occupying the gene's

position in each of the homologous chromosomes. In other words, it describes an individual that has 2 different alleles for a trait. In diploid organisms, the two different alleles were inherited from the parents. Loss of heterozygosity in a cell represents the loss of a single parent's contribution to part of its genome. A common occurrence in cancer, it often indicates the presence of tumor suppressor gene in the lost region. Often, the remaining copy of the tumor suppressor gene will be inactivated by a point mutation. LOH can arise via several pathways, including deletion, gene conversion, mitotic recombination and chromosome loss.

As allelic loss at a certain region of chromosome is thought to indicate the presence of a tumor suppressor gene, LOH analysis is presently the most common method used to identify potential locations for these genes. Although LOH is a common phenomenon in many cancers, a frequent targeting of allelic loss at a specific chromosomal region indicates the location of a tumor suppressor gene. According to this hypothesis, there are several undiscovered tumor suppressor genes which potentially play important roles in cancer development (Macleod, 2000). The genetic changes that result in LOH known to date are mitotic recombination, deletion, gene conversion, translocation, chromosome breakage, chromosomal fusion or telomeric end-to-end fusions, or loss of a whole chromosome (Thiagalingam et al., 2002). Loss of a whole chromosome is generally accomplished by non-disjunction resulting in defects in chromosome segregation (Nigro et al., 1989; Schutte et al., 1996). Mitotic recombination is the result of the non-sister chromatid exchange in mitotic cells (de Nooij-van Dalen et al., 1998; Varella- Garcia et al., 1998). Loss of one single allele or gene may be caused by a deletion resulting from two double-strand breaks or mitotic recombination involving the homologous chromosomal arms. Homozygous deletions within a gene or locus indicate that a double hit may occur targeted to specific chromosomal sites (Hahn et al., 1996; Schutte et al., 1995). In a cell, multiple mechanisms have evolved to ensure the maintenance of genetic material during the processes such as DNA replication and recombination or, to protect the DNA from nucleases and other adversities of intracellular byproducts (Hoeijmakers, 2001). Any defect in these cellular functions could lead to genomic instability, such as loss of heterozygosity.

Literature Review

Oral and esophageal cancer is the fifth most common cancer world-wide and has one of the lowest 5 year survival rates among all cancers (Boring et al., 1992). Despite advances in surgery, radiotherapy and chemotherapy over the past 35 years, there has been no significant enhancement in the survival of oral cavity cancer patients. Improved survival will require identification of reliable prognostic markers that provide a rational basis for assessment of risk for progression. Head and Neck Squamous Cell Carcinoma (HNSCC) is usually regarded as one tumor entity. This seems partially justified due to the fact that most squamous epithelia in the head and neck region, including the entire larynx, hypopharynx and oropharynx, are derived from the endoderm (only the anterior floor of the mouth area and the dorsal and lateral part of the tongue are derived from the ectoderm).

Cell growth and differentiation are two fundamental aspects of multicellular existence, and intertwined with these processes is the phenomenon of unlimited growth which is the basis of the neoplastic state. One of the most promising avenues comes from the recent developments in understanding cell cycle regulation. The molecular regulation of the cell cycle maintains homeostatic balance between cell growth, differentiation, survival and death. Two families of proteins that form complexes play key roles in this process as positive regulators. They are cyclins and cyclin dependent kinases (cdks). The negative regulatory forces are provided by tumor suppressor proteins, notably those encoded by the tumor suppressor genes p53 and Rb (retinoblastoma), and a newly discovered family of proteins known as cdk inhibitors. Most of the molecular level studies have demonstrated that more important alterations in malignancies occurred in negative regulators than in positive ones.

In order to maintain homeostasis, cells must possess negative regulatory mechanisms to balance the growth promoting forces that drive cellular proliferation. Many groups of investigators have uncovered the existence of several distinctive cdk inhibitors, linked to p53, exogenous growth inhibitors and pRb. These inhibitory proteins target the net activities of a broad array of cdks and cyclin/cdk complexes. Studies on tumor suppressor genes have attributed many functions to these genes such as signal

transduction, proliferation, differentiation and apoptosis. Cyclin dependent kinase inhibitors (CKI) are a group of proteins which act as inhibitors of various CDKs and play crucial role mediating extracellular negative signals which result in cell cycle arrest at different G1 points. To date many CKIs have been identified, of which p16 was shown to be involved in regulating the key steps of cell cycle progression and also found to be altered in various malignancies.

Oral cancer is the most common cancer in India and studies at molecular level have shown that the genetic aberrations involved in the development of oral cancer in Indian population have some significant differences from that seen in western countries. These genetic variations in oral cancer samples may be due to differences in etiology (tobacco chewing oral habit is the most predominant in India). However, no molecular genetic studies so far have attempted with the tumor samples from raw betel-nut associated oral and esophageal cancer.

Genetic instabilities are hallmark of human cancers. Genome-wide detection of chromosomal changes, including loss of heterozygosity (LOH) and copy number alterations, either gain or loss, are the focus of substantial attention in cancer research. LOH is frequently observed in a variety of human cancers, and regions with frequent LOH may contain tumor suppressor genes. In addition, LOH may associate with the regions affected by haploinsufficiency and will likely remain a cornerstone for predicting tumor aggressiveness for many human tumors (Maris, 2005). Thus, detection of LOH in oral malignant lesions and its possible predictive values were recently reviewed (Zhang and Rosin, 2001).

Microsatellites are the repeated DNA sequences widely scattered with the biological genome, closely linked to many important genes, and fit for the sensitive polymerase chain reaction (PCR). Therefore, microsatellites are widely used for the genetic linkage analysis and the gene mapping. Since the initial description in 1993 of LOH in colon carcinoma (Thibodeau et al., 1993) microsatellite LOH has been identified widely in a variety of human cancers (Ionov et al., 1993; Aaltonen et al., 1993). Microsatellites also known as STRs (short tandem repeats), are tandem repeats of simple two to six bases that occur abundantly and at random throughout most eukaryotic genomes (roughly one microsatellite every 100 bp long). They are typically

short (often less than 100 bp long) and embedded within unique sequences, thus being ideal for designing flanking primers for *in vitro* amplification by the polymerase chain reaction (PCR). The high degree of polymorphism, as a result of variation in the number of repeats, and the stability displayed by microsatellites make them perfect markers for use in constructing high-resolution genetic maps to identify susceptibility loci involved in common genetic diseases. A microsatellite is analogous to a bookmark; it highlights a particular position in the genome and it is for this reason that their use has become so widespread. Each normal human has 22 pairs of matched (homologous) chromosomes plus a pair of sex chromosome (either XX or XY) each one of the pair of chromosomes is inherited either maternally or paternally and because of normal polymorphism, at any given position (locus) on the chromosome, the DNA sequence can be slightly different; that is they are heterozygous. For example, a normal human cell might have three CA repeats on one allele and five repeats on the other, this marker would be heterozygous.

By analyzing these markers, regions that have been subjected to allelic imbalance, for instance by deletion, can be identified. This will be reflected by the loss or alteration of the markers across the region, that is, the region is tested for LOH or instability. The significance of such somatically acquired genetic changes is well documented and often these manifest as neoplasia.

Some microsatellite loci often reveal the hot spots of LOH at high frequency in some specific malignancies. The tumor suppressor genes, which are related to these cancer development and progression, possibly harbor in the vicinity of these hot spots. Therefore, the study of LOH by microsatellite analysis is an important method to detect the putative tumor suppressor genes. LOH at the chromosomal regions supposed to contain tumor suppressor genes might be related to the process of malignant development, although it is recognized that the development of malignancies, in general, requires multiple genetic alterations (Renan 1993).

Retinoblastoma (Rb) and p16 gene products are part of the Rb pathway that controls the cell cycle. Cell cycle regulation is critical in tumorigenesis and Rb gene inactivation has been reported in many cancers. The Rb gene is located on the long arm of chromosome 13. The Rb protein is a nuclear phosphoprotein that is expressed

in most normal cells. Rb-functions during the G1-S transition within the cell cycle. The hypophosphorylated form of the Rb protein at the G1-S transition is driven by the cyclin-dependent kinases (CDKs), in protein complexes with cyclin D1. Strong inhibitors control these complexes, including the tumor suppressor gene, p16. The p16 gene is located on chromosome 9p21 and its protein blocks cellular proliferation at the G1-S phase of the cell cycle by binding to the CDKs, primarily CDK4 and CDK6. This prevents formation of the catalytically active cyclin D-CDK4 or CDK6 complex, which phosphorylates the Rb protein that drives the cell cycle. The inactivation of p16 and Rb leads to an imbalances of cell cycle regulation and therefore, unrestricted proliferation and tumorigenesis.

In oral carcinogenesis in general, allelic loss of 3p, 9p and 17p is frequent and is a critical genetic event involved in early stage oral cancer (Mao et al., 1996).

Cancers stem from the cell's inability to heed normal growth and division controls or to undergo self-destruction, called apoptosis, when it detects it is damaged. Normal cells are part of a cellular community and co-ordinate their activities with those of their neighbors especially regarding growth and division. Cancerous cells ignore cellular controls and even produce false signals for coercing their neighbors to help them. This errant behavior comes about due to the accumulation of small mutations changes to the cellular genome which is perpetuated in cell reproduction. In the present investigation we have made an attempt to study the deregulation of p16INK4a by deletion mapping using a panel of four polymorphic microsatellite markers selected on the basis of their map position and heterozygosity pattern, from the NCBI website. Oral and esophageal carcinoma (EC) is one of the six most common malignant diseases in the world with a remarkable geographical distribution. The prognosis of EC is very poor; its 5-year survival rate is only about 10% for the patients at late or advanced stage. We have collected all the samples from the North-East part of India where the occurrence of oral and esophageal cancers is very high due to raw betel-nut chewing.

Materials and Methods:

Tissue Samples

A total of 60 tumor biopsies derived from previously untreated primary invasive oral and esophageal carcinomas and the corresponding peripheral blood samples comprised the material for the study. There were no age, ethnicity, sex or stage restrictions on collection of sample. The tissues were ascertained from patients treated at the Nazareth Hospital and North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS) at Shillong after appropriate informed consent and the approval of the protocol by the institutional review board. There are 24 oral cancer samples and 33 esophageal cancer samples. Their histopathological grading was performed by histopathologists. Table 2.1 ?

Table 2.1 Details about the Tissue samples

Samples Collected From	*Number*	*Oral histological details*	*Esophageal histological details*
Nazareth Hospital	38 22 M 16 F	**20** hyperplasia 6; dysplasia 2 Squamous cell carcinoma 12	**18** hyperplasia 2; dysplasia 2 Squamous cell carcinoma 14
NEIGRIHMS	19 15 M 4 F	**4** dysplasia 1 Squamous cell carcinoma 3	**15** dysplasia 1 Squamous cell carcinoma 14

In Total: 17 MO 20 ME; 8 FO 12 FE

The epithelial cells are capable of division so as to maintain a constant epithelial population, as cells are shed from the surface. Tissue homeostasis requires differentiation and desquamation at the epithelial surface to be matched by cell division. The concept of epithelial homeostasis implies that cell production at the deeper layers will be balanced by loss of cells from the surface. When this epithelium is subject or exposed to any irritant or carcinogen such as BNE it causes an inflammation and it leads to an alteration in the balance so that an epithelium may become thicker (hyperplastic) than normal. Hyperplasia is a condition where there is increased cell proliferation but the integrity of the cell is maintained. If this condition persists it leads to a condition where there is increased cell proliferation accompanied with a displacement in the cells from its normal position, which is termed as dysplasia. Dysplasia further leads to carcinoma *in situ.*

Should have been included in the Introduction.

Ref ?

Citation of fig. in the text ?

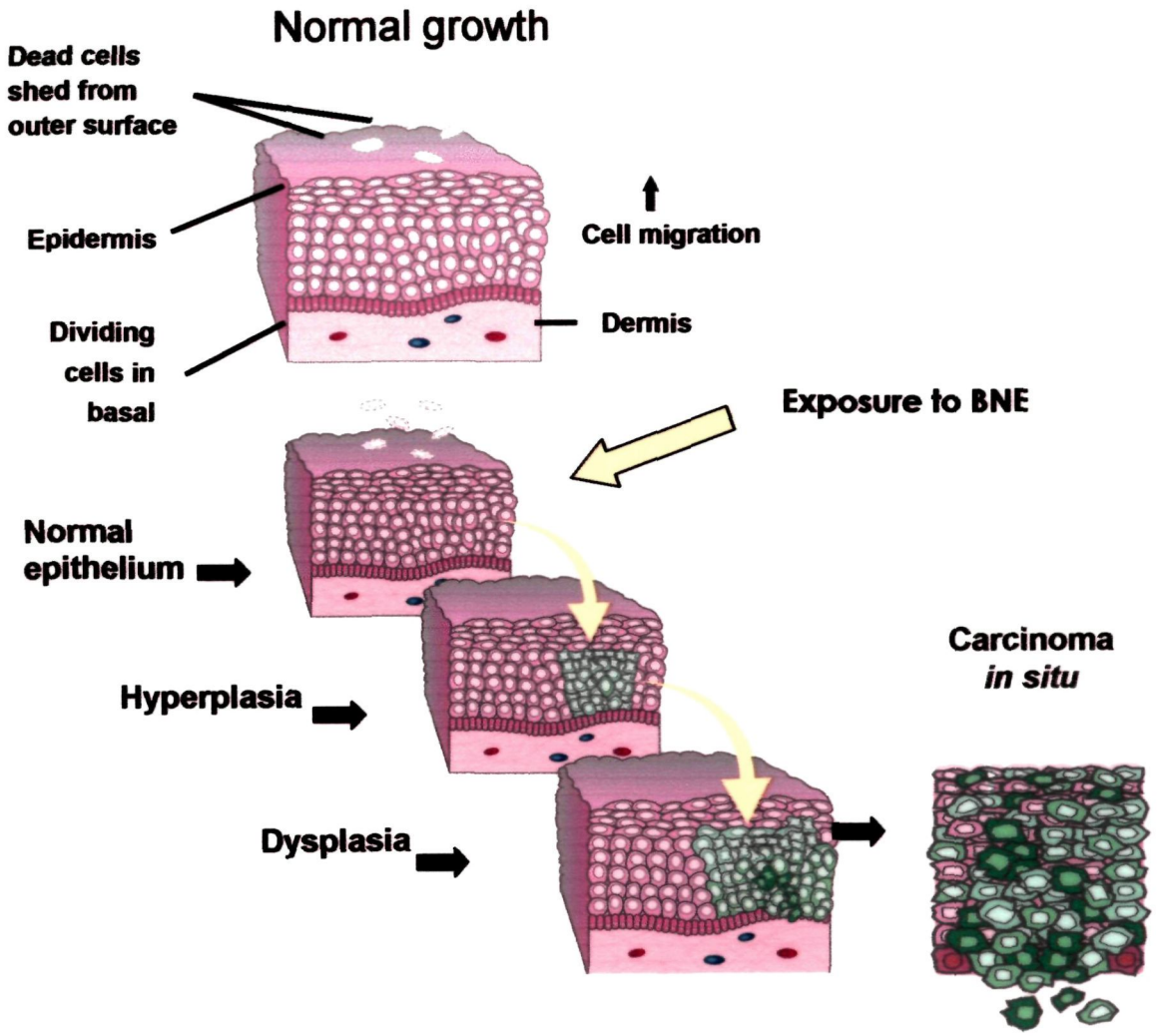


Illustration showing the biology of the normal oral mucosa and Carcinogen / BNE exposed mucosa showing the changes

DNA Extraction Buffers

1X NET Buffer for 100 ml / TNE (Tris-NaCl-EDTA) Buffer

100 mM NaCl = 2 ml of (5M NaCl)

25 mM EDTA = 5 ml of (0.5 M EDTA)

10 mM Tris = 1.0 ml of 1.0M Tris (pH 8.0)

Autoclaved Millipore H_2O = 92ml

TAE Buffer (50X) for 1000 ml

242 gm of Tris base; 57.1 ml of Glacial Acetic Acid

100 ml of 0.5 M EDTA (pH 8.0) Autoclaved Millipore H_2O = 842.9 mL

1X buffer was prepared from the stock solution.

Phosphate Buffer Saline (1X) (PBS) pH7.4

NaCl - 8.0 gm (137mM) KCl - 0.2 gm (2.7mM)

KH_2PO_4 - 0.24 gm (2mM)

Na_2HPO_4 – 1.44 gm (or) $Na_2HPO_4.7H_2O$ – 2.68 gm (10mM)

Add all these to 1000 ml of Autoclaved Millipore water and adjust the ph to 7.4

Lymphocyte Extraction

HISTOPAQUE – 1077 (Sigma, USA). RPMI 1640.(Gibco, USA)

15 ml conical centrifuge tubes. (Greiner)

Procedure

To a 15-ml conical centrifuge tube 5 ml of HISTOPAQUE – 1077 was added and brought to room temperature. Carefully 5ml of blood mixed with RPMI 1640 in equal proportions (2.5ml of blood + 2.5ml of RPMI 1640) was layered on to the HISTOPAQUE – 1077. This was centrifuged at 1200 rpm for 30 minutes at room temperature. After centrifugation the mononuclear opaque ring that was formed at the blood HISTOPAQUE interface was carefully removed by aspiration. The aspirate was resuspended in isotonic solution Phosphate Buffered Saline (PBS) and centrifuged at room temperature at 10,000 rpm for 10 minutes and this procedure was repeated for three times to remove the Histopaque. After the last wash the cell pellet was used for extracting DNA, and for subsequent experiments.

DNA Extraction from lymphocytes

1. The isolated lymphocytes were taken in a microcentrifuge tube and 100-150µl of NET buffer was added
2. 10% SDS (5 µl for 100 µl of NET buffer) and Proteinase K (20 mg/ml) {2 – 2.5µl for 100 µl of NET buffer} was added.
3. The microcentrifuge tube was incubated at 55^0C overnight, and the microcentrifuge tube was intermittently shaken for better digestion.
4. After 8 -10 hrs Proteinase K was added again.
5. After completion of digestion, $1/4^{th}$ volume of 5M NaCl was added.
6. The microcentrifuge tube was kept in ice for 30 minutes to 1 hour.
7. The microcentrifuge tube was centrifuged at 10,000 rpm for 10 minutes at 4^0C. The supernatant was collected in a fresh tube,
8. After centrifugation 700µl of 70% Ethanol was added to the white pellet and the pellet was dissolved completely in ethanol. After complete dissolution again centrifuged at 10,000 rpm for 10 min at 4^0 C.
9. Discard the ethanol, air dry the visible or invisible DNA at room temperature for 10 -15 min.
10. 50 -100 µl of 1X TE (pH 8.0) or autoclaved millipore water was added to the microcentrifuge tube and keep the tube overnight at 37^0 C. the extracted DNA was stored at 4^0 C until use.

DNA Extraction from Tissue

1. The tissue was minced as much as possible using sterile scissors.
2. Minced tissue was taken in 1.5ml micro centrifuge tube, and 100-150µl of NET buffer was added.
3. The tissue was homogenized in NET buffer with tissue grinder.
4. 10% SDS (5 µl for 100 µl of NET buffer), was added and 4-5 µl of Proteinase K (20 mg/ml) was added, and incubated at 55°C overnight.
5. After 8 -10 hrs add again Proteinase K was added.
6. $1/4^{th}$ volume of 5M NaCl was added and incubated in ice for 30 min to 1 hr.

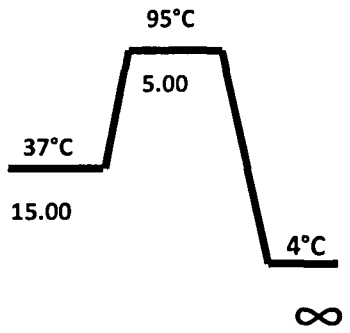
7. After 1hr it was centrifuged at 10,000 rpm for 10 min at 4^0C.
8. After centrifugation 700µl of 70% Ethanol was added to white pellet and the pellet was dissolved completely in ethanol. After complete dissolution again centrifuge at 10,000 rpm for 10 min at 4^0C.
9. Discard the ethanol, air dry the visible or invisible DNA at room temperature for 10 -15 min.
10. Add 50 -100 µl of 1X TE (pH 8.0) or autoclaved millipore water to the micro- centrifuge tube and keep the tube overnight at 37^0 C. Now the DNA was stored at 4^0C until use.

5′-End Labelling of Primers

Nucleic acid were labeled using γ^{32}P. When a phosphorus label is being used the radioactive atom replaces one of the three P atoms in the phosphate group of the nucleotide. For end labeling, in which the attached nucleotide retains its intact phosphate, the P_γ usually the one that is labeled. Polymorphic primers were obtained from Bangalore Genei and 1/5th of the forward primer was labeled with γP^{32}ATP.

Procedure

DTT (1,4- Ditheithretol) 100mM	1µl
10X Kinase Assay Buffer	2µl
Primers (Forward) 20 pmol/µl	2µl
T4 Polynucleotide Kinase (10 units/ µl)	3µl
γ- P^{32} ATP (10 µCi / µl)	4µl



All the components were gently mixed and the tubes were kept in the thermal cycler and run according to the temperature profile as shown above. The tubes were stored in small lead container and stored in refrigerator at 4°C until use. Always β- Shield was used during all the steps during the handling of isotope. .

DNA sequencing dye

95% Formamide; 0.05% Bromophenol blue;

20mM EDTA; 0.05% Xylene Cyanol

20mM NaOH

For 100 ml

95% Formamide - 95ml, 0.05% Bromophenol blue - 50mg,

20mM EDTA-744mg, 0.0.5% Xylene Cyanol - 50mg,

20mM NaOH - 80mg, Autoclaved Millipore Water - 5ml

DNA Gel Loading Dye (6X)

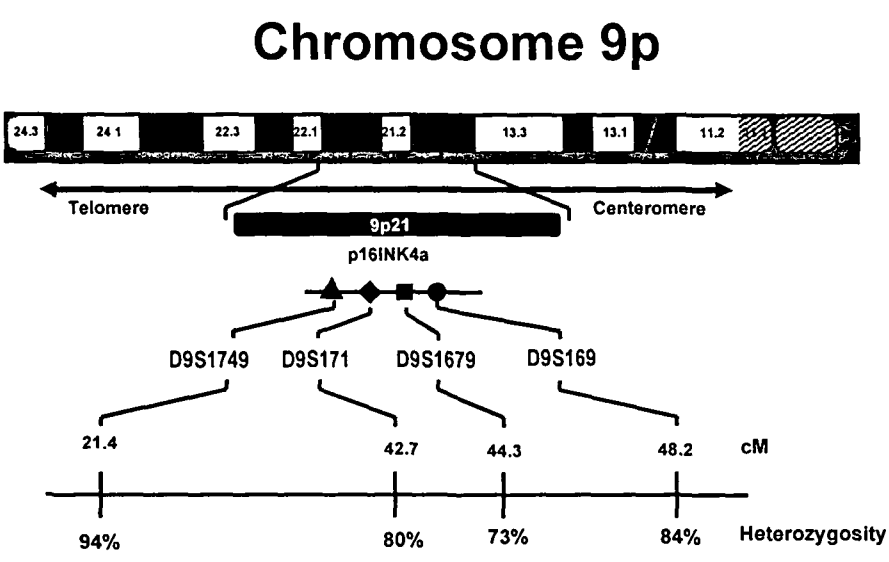
Bromophenol Blue 0.25% (w/v); Sucrose 40% (w/v).

TBE Buffer (Tris Borate EDTA) 10X (1000ml)

Tris Base-108gm; Boric Acid-55gm, $Na_2EDTA.2H_2O$-9.3 gm;

Autoclaved millipore water 1000 ml

1X buffer was prepared from the stock solution and the pH was adjusted to 8.3



Diagrammatic representation of the chromosome map and the position of microsatellite markers and their heterozygosity pattern

Primer Selection and Primer Labeling

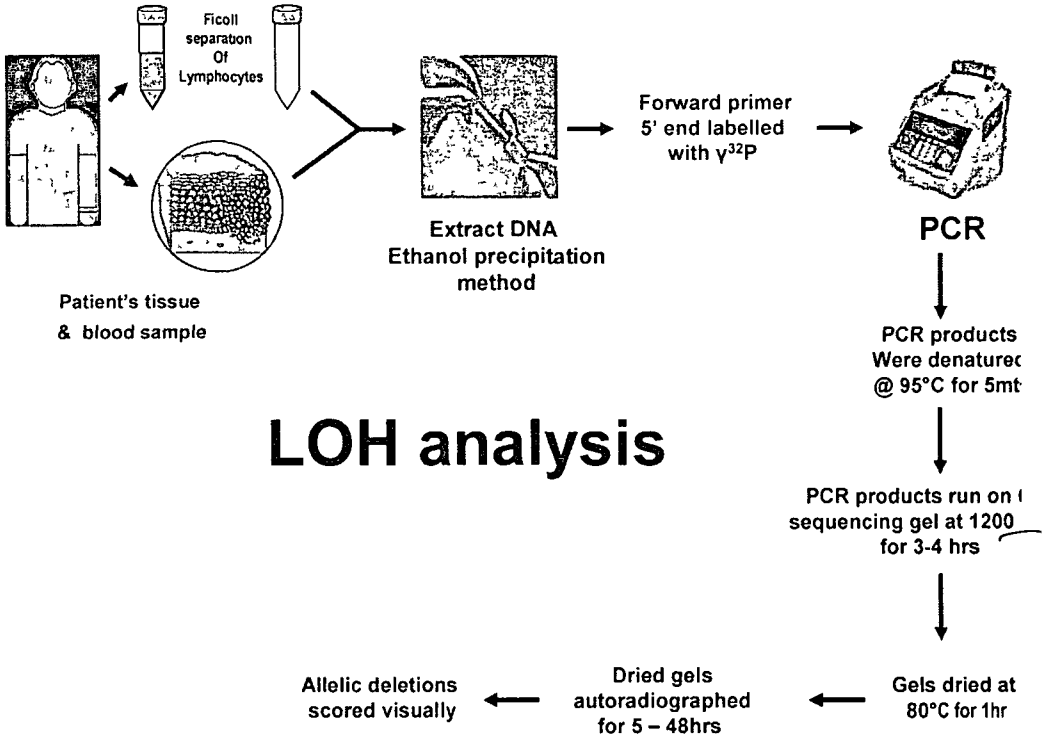
For chromosome 9p, in which p16 gene was being targeted for inactivation in oral and esophageal carcinoma were selected based on their genetic location with a minimum heterozygosity of 0.70 (according to Genome Data Base). STRs used in LOH analyses in this work were D9S1749, D9S171, D9S1679, and D9S169. Several online resources were utilized to obtain polymorphic loci, allele frequencies, primer sequences, and location on genetic maps. The primary online resources used were National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), The Genome Database (http:// gdb.www.gdb.org). Primer optimization was done for each of the primer and was used for subsequent PCR amplification of the samples.

Table 2.2 Primer Details

Microsatellite	PCR product size	cM distance	Heterozygosity (%)	Forward and Reverse primer
D9S1749	120	21.7	94	5' AGGAGAGGGTACACTTGCAA TACAGGGTGCGGGTGCAGATAA
D9S171	159 – 177	42.7	80	5' AGCTAAGTGAACCTCATCTCTGTCT ACCCTAGCACTGATGGTATAGTCT
D9S1679	135 – 145	44.3	73	5' CACCTCTGCCTGCCAA TGCTGTGGACCTAACAAAAA
D9S169	259 – 275	48.2	84	5' AGAGACGATCCAGATCCCA TAACAACTCACTGATTATTTAAGGC

Reagents for PCR

Taq DNA polymerase 3U/µl (Genei)
dNTP mixture 100mM (Genei)
Primers 20 pmols (Genei)
PCR water (Sterile milli-Q)
10xTaqDNAPolymerasebuffer(Genei)
γP^{32} dATP, 10µCi/µl (BRIT, India)
Ezee-6% Sequencing gel (Genei)
Ammonium persulfate (Genei)
10X TBE buffer (Genei);
Acrylease (Stratagems)
Developer (Allied Scientific India)
Fixer (Allied Scientific India)



Time Requirements

Gel preparation: 2 - 3 hours.

High-resolution denaturing polyacrylamide gel electrophoresis: 3 - 6 hours.

Gel drying: 1 hour at 80°C.

Autoradiography: 5 – 48 hours depending on the count.

Preparation of the Glass Plates

Glass plates were cleaned thoroughly with cleaning solution and dried. The glass plates were cleaned again with alcohol. To the small plate small quantity of Acrylease was sprayed evenly and rubbed in a circular motion. The plates were quickly assembled without touching the clean surface. 0.4 mm spacers were placed on the edges of the larger plate. The smaller glass plate was placed on top of the larger plate and spacers. The plates were secured and sealed with sealing tape.

Casting of the Gel

480 µl of 10% ammonium persulfate was added to 75 ml of 6% Ezee sequencing gel mix and mixed gently. The nozzle of the bottle was held at the corner of the gel cast. The gel cast was held at an inclined angle the bench and the gel was poured between the plates and care was taken so that no air bubbles were formed. The straight side of the comb was inserted approximately 1 cm into the gel. This whole setup was left undisturbed and the gel was allowed to polymerize for a minimum of 3-4 hrs.

PCR

The reagents were thawed before beginning the procedure. All reactions were prepared on ice. Polymerase Chain Reaction (PCR) was performed using primer pairs which were selected on the basis of their map position and their heterozygosity pattern from Genome Data Base. PCR was carried out in a 10 µl reaction volume, containing 50-100 ng of genomic DNA with 20 pmols of primer (both forward and reverse) in which one fifth of the forward primer was end-labelled with [γ^{32}P] ATP, and amplified for 25 – 30 cycles.

PCR products were denatured in sequencing stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanole).

Agarose Gel Electrophoresis

PCR products were run in 1.5% agarose gel containing ethidium bromide at 100V for approximately 45 minutes and viewed in Kodak Gel logic 100 imaging system and the image was captured using Kodak imaging software.

Sample loading for sequencing

The gel was fixed on to the sequencing apparatus and 1X TBE buffer was filled both on the upper and lower chamber

The comb was removed gently and the lanes were cleaned to clear of excessive gel that would block proper loading of the sample.

Pre-run of the gel was done for 15-20 minutes.

Gel Loading

The samples were denatured in thermal cycler at 95°C for 5 minutes.

The samples were removed from the thermal cycler and immediately place on ice.

4-5 µl of each sample was loaded per well.

The gel was run at 1200 volts for 3-5 hours (running time based on PCR product size).

Separation of Gel

The power supply was turned off and the buffer was drained from the upper chamber. The glass plated assembly was removed from the sequencing apparatus. The plates were removed by inserting a spatula in-between the glass plates. Whatman paper was placed on the gel, and the Whatman paper was slowly removed along with the gel off the glass plate. The gel was covered with saran wrap and dried on a gel dryer for 1 hour at 80°C.

Autoradiography and Development

The dried gel was placed in the film cassette and photographic film was placed on top of the film in the dark room. The film was exposed for 5 – 48 hours depending on the count.

The film was removed after appropriate exposure time and then developed by immersing the film in developer for 5 minutes and then rinsed in tap water. The film was placed in fixer for 5 minutes and then rinsed in tap water. The film was air dried and then the allelic deletions were scored visually.

Results

The samples for the study were from two places, Nazareth hospital, and NEIGRIHMS of Shillong (Table 2.1). Out of these 60 samples taken for study 2 samples showed histopathologically negative and one sample did not amplify. Therefore, out of total 57 samples 64% were males and 36% were females. In this study population the age distribution ranged from 27 to 86 with the mean age being 51.4 years. 42% of the cases were oral cancer and 58% of the cases were esophageal cancers. Out of total 57 samples, 11 samples were hyperplasia, 9 samples were dysplasia and the rest are squamous cell carcinoma. Two samples showed oral cancers without betel-nut chewing and smoking habit, whereas 5 cancer samples showed only smoking habit and 15 samples showed cancers in patients who have only betel-nut chewing habit. Rest cancer patients showed habit of both betel-nut chewing as well as smoking.

Figure 2.1 shows the agarose gel pictures of the PCR amplified products of four different microsatellites used in our study.

Figure 2.2 represents the LOH status of 57 tumor samples used for the study. Table 2.3 represents the frequency of LOH which varied among the markers from 21% (D9S171), 28% (D9S196), 30% (D9S1679) and 40% (D9S1749). The analysis revealed deletions in at least one locus in 33 tumors (58%) showed LOH at one or more loci of 9p while others retain heterozygosity at the remaining loci, suggesting a regional loss. The chewing habit of the patients who showed LOH was that, 1 did not chew betel nut, 3 chewed less than 10, 14 chewed 10 pieces or less and 15 chewed more than 10 (ranging from 10 to 40) pieces of betel-nut per day. Besides betel-nut chewing, 12 male and 1 female patients were smoking cigarette more than 10 per day whereas 8 male patients were smoking 5 per day and 12 female patients did not smoke any cigarette. Present data show that only tobacco smokers (only 4 samples) did not show any loss in 9p region, whereas only BNE-chewers showed LOH mainly in the 9p region.

Evaluation of LOH of 57 invasive oral and esophageal cancers with 4 sequence-tagged repeat polymorphic markers mapped to chromosome 9p revealed deletions

in at least one marker in 58% (33 cases) of the tumors. Of the 33 tumors that had LOH on 9p, 4 (12%) showed LOH at all of the informative markers, suggesting 9p monosomy. The pattern of LOH in the tumor samples that exhibited regional losses on 9p was used to identify minimal regions of deletion (Fig 2.2). This LOH pattern in invasive cancers revealed one common region of minimal deletions at 9p21.1 The 9p21.1 minimal region of deletion derived from 22 tumors spanned the marker D9S1679 flanked by marker D9S171 proximally and D9S169 distally. The deletion boundaries were defined by tumors T28, T58, T76, T94 and T100 proximally and tumors T51, T54, T28 and T60 distally. The 9p21.3 minimal deletion was deduced from the pattern of LOH in 21 tumors as shown in (Fig 2.2 and 2. 3). This deletion was spanned by only one marker D9S1749 and thus more markers have to be introduced to define the minimal region of deletion in this region.

Relationship between LOH and clinicopathological features

The samples taken for study were histologically classified into hyperplasia (18%), dysplasia (10%) and squamous cell carcinoma (65%). Out of 33 tumors that showed loss at 9p21, 20 are esophageal tumors and only 13 are oral tumors. Of 33 individuals with cancer, 33% were moderate, and 36% were well differentiated SCC. 6% were dysplastic and 24% were classified histologically as hyperplasia. Out of the 57 informative samples 33 tumors showed loss in atleast one of the microsatellite markers. Out of these 33 samples 20 were from male patient and only 13 were from female.

Table 2.4 shows the demographic data which reveal that the age of onset of cancer is lowered (<50 yrs) when the subjects under study were found to smoke 5-10 cigarettes per day along with chewing betel nut.

Figure 2.3 shows the region of minimal deletion which is defined as the region that is found to be deleted in most of the samples. Here the data reveals that there

is a region of deletion spanning about 4cM between the microsatellite markers D9S1679 and D9S169.

Figure 2.4 is a representation of gels showing loss of heterozygosity (LOH) in betel associated oral and esophageal carcinoma. The 9p21 region of deletions is represented by three tumors, T51, T69 and T64. Tumor T51 shows LOH of 9p21 marker D9S1679 and D9S169 but retain heterozygosity of proximal marker D9S171. Tumor T69 and T64 show LOH of 9p21 marker D9S171 and D9S1679 but retain heterozygosity of distal marker D9S169.

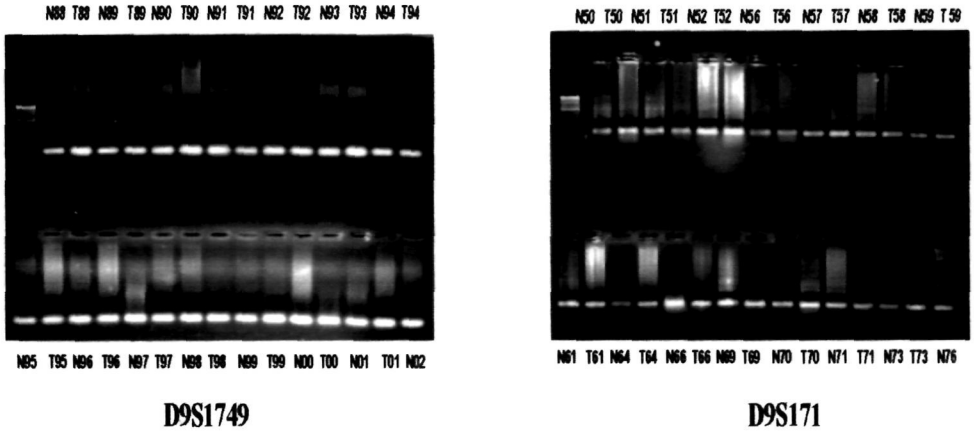


Figure 2.1 Representation of the agarose gel pictures of the PCR amplified products of the four different microsatellite markers.

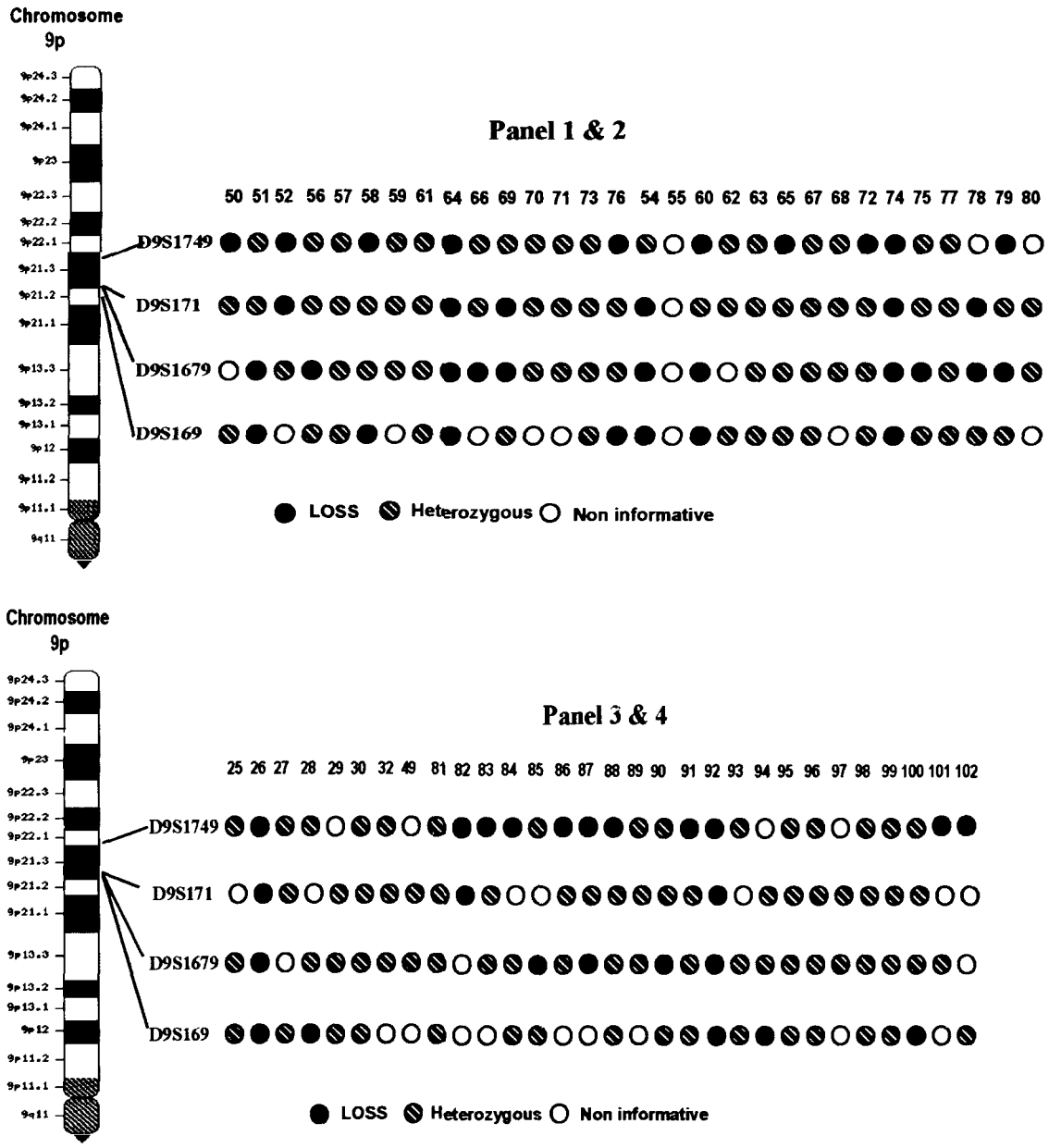


Figure 2.2 LOH status of Panel 1 to 4.

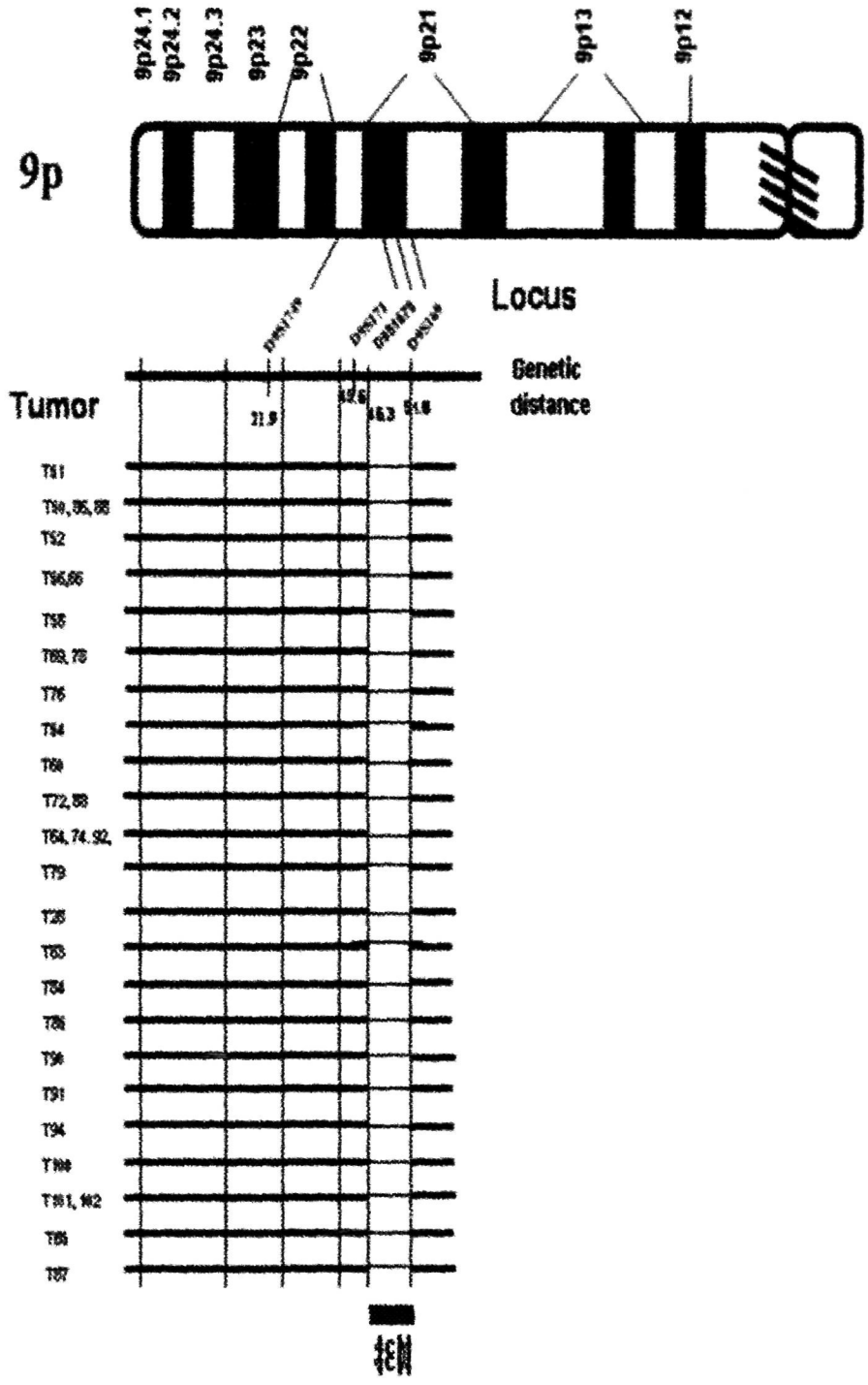


Figure 2.3 LOH results for the microsatellite markers D9S1749, D9S171, D9S1679 and D9S169.

Table 2.3
Summary of Frequency of LOH on chromosome 9p in oral and esophageal carcinoma

Chromosome Band	Locus	Genetic position cM	Heterozy-gosity	# LOH / informative	LOH %
9p21	D9S1749	21.74	94	21 / 53	40
	D9S171	45.57	80	09 / 52	21
	D9S1679	46.25	73	16 / 54	30
	D9S169	49.65	84	12 / 43	28

cM: centimorgan **LOH:** loss of heterozygosity

Table 2.4 Demographic data regarding the oral and esophageal cancer samples

No. of samples	M / F	Age 35-50	Age 51-80	Habits BNE / CS	Cancer type Oral	Cancer type Esopg	Status	Status of Loss at 9p
1	0 / 2	0	2	N / N	2	0	Dys1/SCC2	2
5	4 / 1	1	4	N / 5–10	3	2	Hyp2 / SCC3	1
15	0 / 15	6	9	10-40 / N	9	6	Hyp3 / /Dys3 / SCC9	9
13	21 / 8	* 11	10	10-40 / <10	7	6	Hyp2 / Dys2 / SCC9	7
22	21 / 1	11	11	10-40 / >10	6	16	Hyp4 / Dys3 / SCC15	13

*2 patients were 28, 27 years old
BNE: betel-nut extract **CS:** cigarette smoking

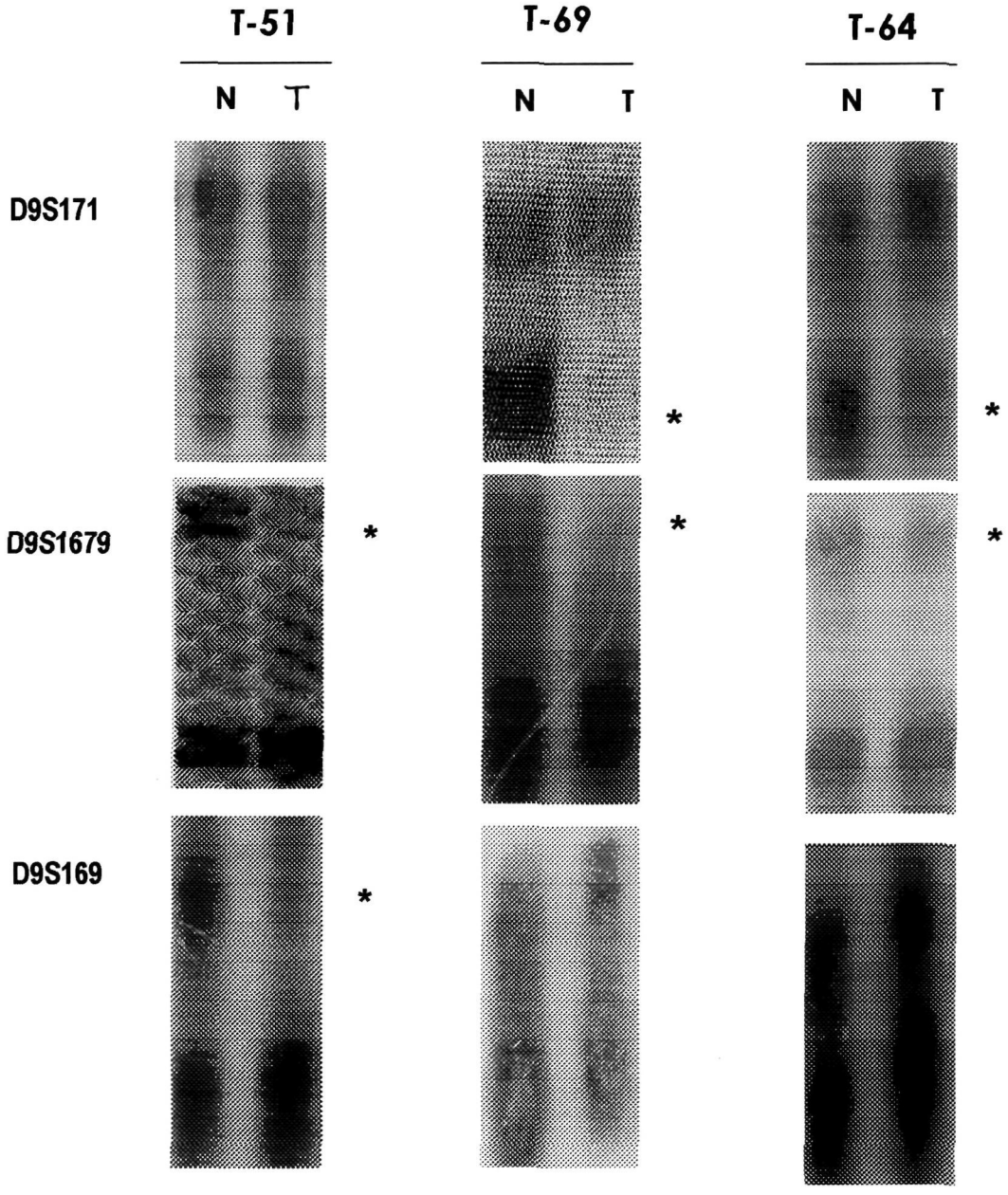


Figure 2.4 Representative gels showing loss of heterozygosity (LOH) in betel associated oral and esophageal carcinoma.
N: normal control DNA; T: Tumor DNA; Asterix: LOH.

Discussion

The molecular genetic mechanisms responsible for betel-associated oral and esophageal carcinogenesis are poorly understood. Factors believed to contribute to the etiology of oral and esophageal cancers include tobacco, betel chewing and alcohol besides age, gender, ethnicity and aspirin use (Kaur et al., 1994). Several studies in the high risk regions of China have demonstrated a strong tendency towards familial aggregation or tendency towards clustering of cases within families, suggesting that genetic susceptibility may play an important role in the etiology of esophageal cancer in these populations. Many previous studies on betel-associated oral cancers focused on alterations of p53 using immunohistochemical staining and p53 mutational analysis Their results were variable, but generally the mutational; frequency of p53 tended to be lower than conventional squamous cell carcinoma (SCC) among western countries (Kaur et al., 1998). In case of esophageal cancers previous studies revealed multiple genetic alterations, including loss of tumor suppressor genes (TSG) and activation of oncogenes, associated with the development of esophageal cancers. Chromosomal regions with frequent allelic loss may point to major TSG that can assist our understanding of the molecular events involved in the development of esophageal cancer and may serve as the basis for development of markers for genetic susceptibility testing or screening for early detection of this tumor.

9p21 is one of the most frequent regions for allelic loss in head and neck SCC (Kresty et al., 2002) and oral precancerous lesions (Nunn et al., 1999). Pande et al., (1998) has analyzed the expression of p16 and Rb in oral malignant tissues and found lack of expression of Rb and p16 in 66% and 63% of the cases respectively.

To better understand the genetic changes involved in the development of oral and esophageal cancer and ascertain potential susceptibility genes, we had conducted a loss of heterozygosity study on chromosome 9p21 regions using 4 polymorphic microsatellite markers. We have screened 57 tumor samples of oral and esophageal squamous cell carcinoma for LOH analysis. In that scan, we found

microsatellite markers having high frequency of LOH. The analysis revealed that deletions in at least one locus in 33 tumors (58%) showed LOH at one or more loci of 9p while others retain heterozygosity at the remaining loci, suggesting a regional loss. This study reveals that loss of microsatellite locus at 9p21 region in oral and esophageal squamous cell carcinoma in the studied population especially in the local tribal population suggests the involvement of the tumor suppressor gene p16 in this region. By comparative genomic hybridization, (Lin et al. 2002) reported on the frequent gains of 8q, 9q, 11q and the frequent losses of 3p, and 4q in Taiwanese betel-associated carcinoma cases. The molecular and clinicopathological characteristics of tumors support a distinct mechanism for oral and esophageal tumorigenesis, which involves the accumulation of various alterations. Concomitant loss of p16, Rb, and p53 are also very important aspects in the progression of oral and esophageal cancer. The results indicate that chewing of betel nut is a major factor for the development of oral and esophageal cancer. In Taiwan and India, most of these patients were male and also had tobacco and drinking habit. Genetic alterations in betel associated tumorigenesis may be affected by the synergic effects of tobacco and alcohol as well as a wide variation in ethnic background, in addition to other habits, gender, and the betel preparation. Vietnamese betel-chewing habit is unique among other countries because only elderly female tend to take it up, and betel quid containing the areca nut, lime paste and betel-leaf without tobacco and other constituents is introduced into the mouth. Frequent promoter hypermethylation of RASSF1A and $p16^{INK4A}$ and infrequent allelic loss other than 9p21 in betel-associated oral carcinoma in a Vietnamese non-smoking / non-drinking female population was reported (Tran et al., 2005).

Cigarette smoking was also indicated as a strong risk factor which acts as a synergistic agent in the pathogenesis of oral and esophageal cancer. The development of oral and esophageal carcinoma is believed to be a multistep process, in which genetic and epigenetic events accumulate as a result of chronic exposure to environmental carcinogens, resulting in loss of cell cycle control, selected cell growth, and finally the development of cancer. Allelic loss of 3p

and/or 9p was reported to be an essential step in the early development of head and neck cancer (Mao et al., 1996). The present data reveal that 9p21.1 region, which harbors p16INK4a, is frequently deleted in dysplastic, hyperplastic oral and esophageal samples and this indicate that loss at 9p21 is an early and critical genetic event in betel associated oral and esophageal carcinogenesis.

From the demographic data it is shown that smoking of tobacco has an influence on the onset of cancer since a good number of patients were affected with cancer below 50 years of age. Therefore, there seems to be a correlation with the habit of chewing along with smoking which was found to act synergistically in enhancing the onset of cancer at an early age. In this investigation, there were 12 female patients who had no habit of smoking or tobacco chewing and all of them showed loss of 9p21 region. On the other hand there were 5 patients who had no habit of chewing raw betel-nut.

Samples which did not show any LOH at 9p21 region may have loss either any other site of chromosome 9p or in any other chromosomal arms. However, aberrant promoter hypermethylation of TSGs is a major epigenetic mechanism for silencing TSGs. Methylation is the main epigenetic modification in humans and changes in methylation patterns play an important role in tumorigenesis. In particular, hypermethylation of normally unmethylated CpG islands in the promoter regions of many tumor suppressor genes correlates with loss of expression (Baylin et al., 1998). The TSG known as $p16^{INK4a}$ exhibits frequent hypermethylation associated gene silencing in many human cancers including bladder cancer (Valenzuela et al., 2002), gastrointestinal cancer and cervical cancer (Rocco and Sidransky 2001). The hypermethylation of $p16^{INK4a}$ has been reported in oral carcinoma associated with betel chewing in Vietnamese patients (Tran et al., 2005). The unique aspect of Tran's study is that all the patients were non-smoking and non-drinking females and therefore they were purely and predominantly betel-related genetic alterations without interference from other confounding factors.

Carcinogenesis might not be the result of just one mechanism of gene suppression. Rather than focus only on loss of heterozygosity of tumor-suppressor

genes, many studies have investigated the role of the combination of loss of heterozygosity and of methylation. The incidence of promoter methylation is enhanced in those tumor suppressor genes that do not have loss of heterozygosity as a prominent mechanism. Because genes can be silenced through at least two mechanisms, promoter methylation might explain why studies have shown widely different results of methylation status, since loss of heterozygosity could vary within their study samples. Various genes have been implicated in squamous cell carcinoma of the oral cavity with differing results. However, methylation is important in the development of oral squamous-cell carcinoma, and other tumor suppressor genes targeted by promoter methylation will no doubt be described in the future. Oral and esophageal biopsies that morphologically represent premalignant lesions already possess genetic aberrations commonly found in end-stage oral cancer. Characterization of INK4a/ARF inactivation events in populations at increased risk for malignant transformation may provide insight into which alterations or combinations of inactivation events are most critical for cancer development.

9p21 region which harbors the TSG p16INK4a has been the focus of this study with regard to its potential role in tumorigenesis. Inactivation of TSG appears to be one of the genetic mechanisms involved in the development of oral and esophageal cancer. This process includes mutation of one allele, followed by a deletion of the remaining one LOH or homozygous deletion of both alleles. Allelic deletions detected as LOH have been proved useful for mapping regions of DNA that contain TSGs. On chromosome 9p21, the INK4a locus encodes both p16 and p14ARF proteins by alternative splicing (Duro 1995). Although the role of p14ARF as a true tumor suppressor has yet to be characterized, p16 fulfills all the criteria for a bona fide tumor-suppressor gene, even judged with the strictest measures (Rocco et al., 2001).

The human p16 protein contains 156 amino acids and was first discovered in a yeast two-hybrid system to detect proteins that interact with human cyclin-dependent kinase 4 (Ruas et al., 1998). The tumor suppressor function of p16 is attributed to its ability to inhibit the catalytic activity of the cyclin-dependent

kinase 4–6/cyclin D complex that is required for phosphorylation of retinoblastoma protein (Serrano et al., 1993 and Serrano 1996). The E2F family of transcription factors is essential for cell cycle progression, and their transcriptional activity is inhibited through the specific binding to retinoblastoma protein b (Hengstschlager et al., 1996). This retinoblastoma protein/E2F complex will release E2F transcriptional factors upon phosphorylation of retinoblastoma protein by cyclin dependent kinase 4–6/cyclin D complex and promote cell cycle progression past the G1/S checkpoint (Rocco et al., 2001, Hengstschlager et al., 1996). By binding to cyclin-dependent kinases 4 and 6 and inhibiting retinoblastoma protein phosphorylation, p16 prevents the formation of cyclin-dependent kinases 4–6/cyclin D stimulatory complex and promotes the formation of retinoblastoma protein/E2F repressive complex, with the end result of blockage of cell cycle progression at the G1/S checkpoint (Rocco et al., 2001).

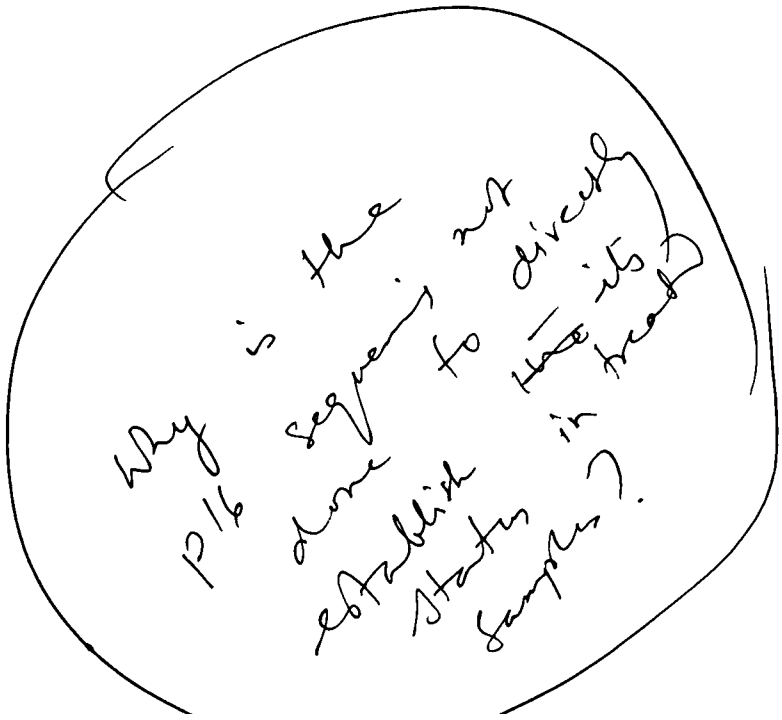
However, before reaching any final conclusion more markers should be selected to screen the whole 9p and then one can identify the region/s of minimal deletion.

SUMMARY

Areca nut or betel nut, the seed of the tall, slender areca palm (areca catechu), native to the fringes of the Indian and west Pacific Oceans. It is thought to have originated on the Malay Peninsula. Areca nut is one of the most widely used psychoactive substances with several hundred million users worldwide, predominantly in southern Asia. It has been estimated by Boucher et al., in 2002 that approximately 600 million people in South-east Asian countries are addicted to areca. Betel users discovered that the addition of lime helps to extract the vital essence of the nut; the catalytic lime is either powder (calcium oxide) or paste (calcium hydroxide). The International Agency for Research on Cancer (IARC) regards betel nut to be a known human carcinogen. In countries and communities where betel is consumed extensively, oral cancer forms up to 50% of malignant cancers. The lime that betel nut is frequently chewed with causes a great deal of the mouth, cheek and tongue cancer. Betel-nut chewers with faulty gene have higher risk of mouth cancer. Oral squamous cell cancer is the most common malignant tumor caused by chewing of betel nut. Powdered slaked lime applied to the chewed Areca nut with Piper betle inflorescence while chewing causes pH to rise to approximately 10, at which reactive oxygen species are generated from betel quid ingredients in vitro. Reactive oxygen species, together with sustained lime-induced cell proliferation, suggest a possible mechanism of carcinogenesis for this tumor. This causes the betel quid ingredients to generate free radicals which, together with carcinogens from the tobacco smoke, cause DNA damage that cannot be repaired because of the rapid cell turnover caused by the caustic lime. The evidence for the carcinogenicity of humans to betel quid with tobacco was evaluated as sufficient; the evidence for betel quid without tobacco was evaluated as inadequate. Epidemiological studies have elucidated that the habit of chewing betel quid (BQ) is associated with an increased risk of oral submucous fibrosis (OSF) and oral cancer (IARC 1985, Kwan 1976). The Khasis in Meghalaya chew one quarter of the betel nut along with apportion of the betel leaf on which lime has been smeared. As a rule the inclusion of tobacco and spices or other performs are avoided. This unique situation favors an investigation into the length and exposure of the oral mucosa to the chemicals released from the BNE

and leaf during the course of the day (Stich et al., 1983). East Khasi Hills a district in Meghalaya of the north-eastern region of India has one of the highest incidence rates of oral, oropharyngeal and esophageal cancer which is of particular concern. Studies reveal that Head and neck cancers still have a high mortality rate.

Oral cancer is the most common cancer in India and studies at molecular level have shown that the genetic aberrations involved in the development of oral cancer in Indian population have some significant differences from that seen in western countries. These genetic variations in oral cancer samples may be due to differences in etiology (tobacco chewing oral habit is the most predominant in India). However, no molecular genetic studies so far have attempted with the tumor samples from raw betel-nut associated oral and esophageal cancer. Genetic instabilities are hallmark of human cancers. Genome-wide detection of chromosomal changes, including loss of heterozygosity (LOH) and copy number alterations, either gain or loss, are the focus of substantial attention in cancer research. LOH is frequently observed in a variety of human cancers, and regions with frequent LOH may contain tumor suppressor genes. Some microsatellite loci often reveal the hot spots of LOH at high frequency in some specific malignancies. The tumor suppressor genes, which are related to these cancer development and progression, possibly harbor in the vicinity of these hot spots. Therefore, the study of LOH by microsatellite analysis is an important method to detect the putative tumor suppressor genes. For our study LOH analysis on chromosome 9p21 region has been conducted on microsatellite markers selected on the basis of their map position and heterozygosity pattern, from the NCBI website. One of the goals of the present study was to investigate the extent of the initial damage or lesions in cells treated with BNE. The effect of BNE was studied both in vivo and in vitro mammalian systems. The *in vivo* study was conducted in mouse BMC and the *in vitro* study was done in human peripheral blood lymphocytes (HPBLs). Both the systems are well established and suitable for the assessment of cytogenetic effects.


Why is the
16 sequencing not
done to directly
establish its
status in treated
samples?

- ✓ In vitro study conducted in HPBLs reveals there was a marginal increase in the frequency of chromatid breaks at 4°C than at 37°C and accordingly the delay in cell cycle kinetics was higher at 4°C than at 37°C. Therefore, it is clear that BNE does not induce initial DNA lesions substantially higher in frequency.

- ✓ The important observation made in the study was that there was an increase in the frequency of CA in mouse BMCs treated with both lime and BNE. Our study shows that BNE mainly induced chromatid breaks and this induction was considerably modulated by depletion of GSH by BSO.

- ✓ A novel aspect in the present study is the treatment of BNE and lime to the mouse BMCs which showed an induction of premature anaphase separation besides chromatid breaks. The frequency of premature anaphase separation was 5% after 30 days of BNE treatment. This frequency was enhanced significantly when BNE and lime was treated to BSO mediated GSH-depleted cells and the frequency was found to increased to 11% after 30 days and 15% after 45 days.

- ✓ 57 oral and esophageal squamous cell carcinoma tumor samples were screened for LOH analysis. In that scan, we found microsatellite markers having high frequency of LOH. The analysis revealed that deletions in at least one locus in 33 tumors (58%) showed LOH at one or more loci of 9p while others retain heterozygosity at the remaining loci, suggesting a regional loss. This study reveals that loss of microsatellite locus at 9p21 region in oral and esophageal squamous cell carcinoma in the studied population suggests the involvement of the tumor suppressor gene p16 in this region.

- ✓ Cigarette smoking was indicated as a strong risk factor which acts as a synergistic agent in the pathogenesis of oral and esophageal cancer. The demographic data reveals that smoking of tobacco has an influence on the onset of cancer since a good number of patients were affected with cancer

below 50 years of age. There seems to be a correlation with the habit of chewing along with smoking which was found to act synergistically in enhancing the onset of cancer at an early age.

Samples which did not show any LOH at 9p21 region may have loss either any other site of chromosome 9p or in any other chromosomal arms. However, aberrant promoter hypermethylation of TSGs is a major epigenetic mechanism for silencing TSGs. The incidence of promoter methylation is enhanced in those tumor suppressor genes that do not have loss of heterozygosity as a prominent mechanism. Because genes can be silenced through at least two mechanisms, promoter methylation might explain why studies have shown widely different results of methylation status, since loss of heterozygosity could vary within their study samples. Various genes have been implicated in squamous cell carcinoma of the oral cavity with differing resul

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